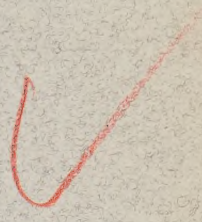


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Weitere Beiträe zu "Chemischen Untersuchungen der Zahnpulpa"

Ueber die Freien Aminosäuren in Zahnpulpa

Von SEIICHI MOTOMURA

(Aus dem Biochemischen Laboratorium der Pharmazeutischen
Frauen-Hochschule zu Kobe, Kobe*)

(Der Schriftleitung zugegangen am 23. Januar 1961)

Im Jahre 1941 hat Shizuka Motomura (1), unter Anleitung von Prof. M. Tomita, mit Versuchsmaterial von 3.2 kg. Schweinezahnpulpen die Fermentwirkung von Glukogenase, Saccharase, Tributylinase, Katalase, Glycerophosphatase und Fructosemonophosphatase nachgewiesen und die in Pulpe vorkommenden organischen sowie anorganischen Substanzen untersucht.

Er hat dabei berichtet, dass von den organischen Substanzen die alkohol/ätherlöslichen und -unlöslichen Anteile 1.14% bzw. 5.16% betragen, und anorganische Substanzen 3.84%, Wasser 89.84%, und der Aminostickstoff des enteweissten Filtrats 0.0008% betrage.

In 1955 untersuchte Jiro Kimura (2) je 0.5~1 g. von Menschen-, Rinder-, Hunde- und Schweinezahnpulpen, indem er sie mit 20.5 %iger Salzsäure hydrolysierte und durch zweidimensionale Papierchromatographie (mit Phenol und Lutidin) entwickelte. Er konnte in dem Hydrolysaten Asparaginsäure, Glutaminsäure, Lysin, Arginin, Histidin, Cystin, Serin, Threonin, Alanin, Valin, Methionin, Glycin, Prolin, Leucin und Phenylalanin nachweisen. Aber dabei wurde kein Fleck von Methionin und Prolin bei Schweinepulpe und kein von Prolin bei Hundepulpe nachgewiesen.

Um die Untersuchung der Zahnpulpe von Shizuka Motomura weiterzutreiben, hat der Verfasser die in ihnen vorkommenden freien Aminosäuren untersucht.

METHODIK

Material—4415 g. von Zahnpulpen wurden frisch

von 736 Rinder entnommen und in Alkohol eingetaucht. Nach sieben Monaten wurden sie vom Alkohol getrennt und mit warmem Wasser 4mal extrahiert. Die vereinigte Lösung von Alkohol- und Wassereextrakt wurde unter vermindertem Druck von Alkohol befreit und eingengt.

Fraktionierung der freien Aminosäuren—Das eingengte Extrakt der Rinder-Zahnpulpen wurde mit der Tannin-Blei-Methode enteweisst. Das von Blei befreite Filtrat wurde durch eine Säule von Dowex-50 (H-Form) behandelt, um alle Aminosäuren zu isolieren.

Papierchromatographie—Für Papierchromatographie wurden folgende Lösungsmittel gebraucht: *n*-Butanol: Essigsäure: Wasser (Abkürzung, Bu/Ac) 80: 20: 20, Pyridin: Butanol: Wasser (Py/Bu) 80: 20: 20, Pyridin: Wasser (Py) 65: 35, Phenol (Phen), gesättigt mit einer wässrigen Lösung von 6.3% igem Na-Citrat und 3.7% igem saurem Ka-Phosphat, und 95% iger Ethanol (Et).

Papier, Toyo Nr. 50.

Nachweis von Aminosäuren durch 0.2%ige Ninhydrin-Lösung in Butanol. Arginin durch Sakaguchi'sche und Histidin durch Pauly'sche Reaktion.

Zur Identifizierung der einzelnen Aminosäuren wurden betreffende reine Aminosäuren mit den Proben nebeneinander chromatographiert.

RESULTATE UND BESPRECHUNGEN

Die aus Rinderzahnpulpen isolierten und an Dowex-50 adsorbierten Aminosäuren wurden mit 0.1 N wässr. Ammoniak eluiert und mit Amberlite IRC-50 bei pH 4.8 säulenchromatographisch in zwei Fraktionen von basischen bzw. von neutralen und sauren fraktioniert. Die beiden Fraktionen wurden weiter in je zwei Fraktionen fraktioniert, und zwar die basische mit Amberlite IRC-50 bei pH 7.0 in Fraktion I und II, und die saure mit Amberlite IR-4B bei pH 7.0 in Fraktion III und IV. Das Fraktionierungsverfahren wurde schematisch in Abb. 1 gezeigt.

*Direktor: Prof. Dr. M. Tomita

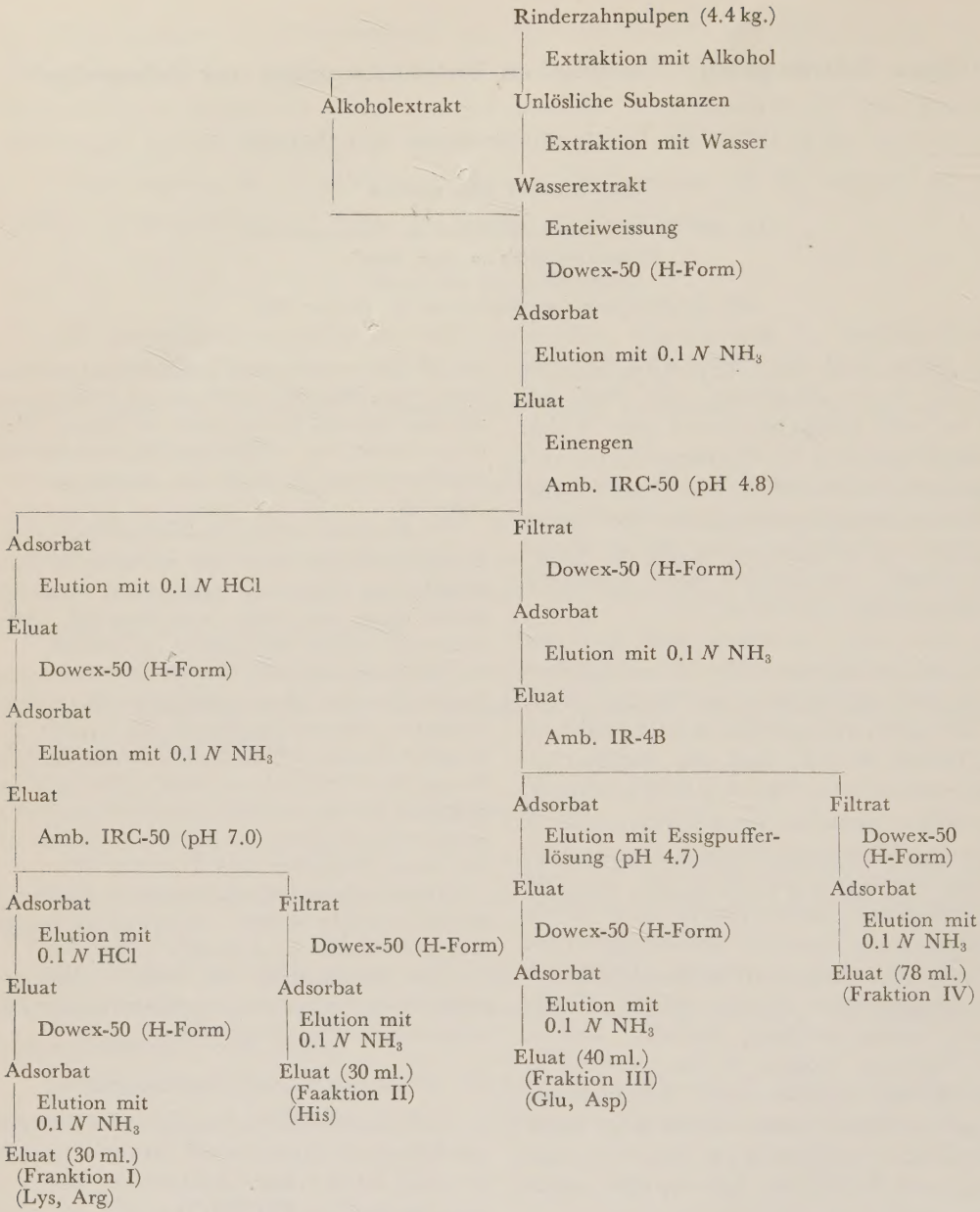


Abb. 1. Fraktionierungsverfahren der Extrakte von Rinderzahnpulpen.

Dann wurden alle Fraktionen papierchromatographisch untersucht. Wie in Tabelle I gezeigt ist, wurden in Fraktion I Arginin und Lysin, in Fraktion II Histidin und in Fraktion III Asparagin- und Glutaminsäuren durch die Versuche mit verschiedenen Lösungsmitteln nachgewiesen.

In Fraktion IV wurden mehrere neutrale Aminosäuren nachgewiesen. Da die R_f -Werte häufig Schwankungen zeigen, wurde weitere Fraktionierung und zwar Isolierung einzelner Aminosäuren erzielt. Zuerst wurde Fraktion IV durch eine Säule von Dowex-50 adsorbiert und mit 0.1 N NH₃ eluiert, um in 27 Unter-

TABELLE I

R_f-Werte der Versuchssubstanzen und der massgebenden reinen Aminosäuren mit verschiedenen Lösungsmitteln

	Bu/Ac		Py/Bu		Py		Phen	
Fraktion I			0.05		0.32	0.38	0.60	0.65
Arginin			0.05			0.38		0.65
Lysin			0.05		0.32		0.60	
Fraktion II			0.10		0.50		0.69	
Histidin			0.11		0.50		0.70	
Fraktion III	0.12	0.18	0.04	0.15			0.15	0.26
Asparaginsäure	0.12		0.04				0.15	
Glutaminsäure		0.18		0.15				0.26

fraktionen zu verteilen. Dadurch kam aber keine scharfe Trennung der einzelnen Aminosäuren zustande, wie es aus dem in Abb. 2 gezeigten Papierchromatogramm ersichtlich ist. Daher wurden die einzelnen Aminosäure durch Papierchromatographie getrennt. Zuerst wurden die oben gewonnenen 27 Unterfraktionen wieder in fünf Fraktionen zusammengesetzt und zwar waren die Zusammensetzungen folgende: 1. Fraktion von 1. bis 7. Unterfraktion, 2. Fraktion von 8. bis 12., 3. Fraktion von 13. bis 19., 4. Fraktion von 20. bis 25. und 5. Fraktion von 26. bis 27.

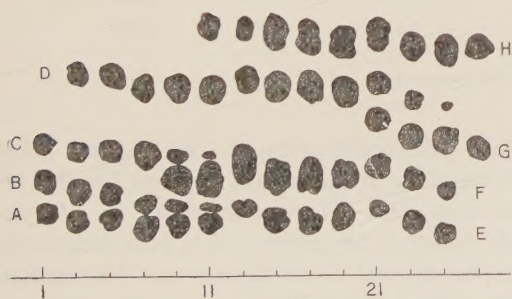


Abb. 2. Chromatogramm der Unterfraktionen von Fraktion IV mit Bu/Ac.

A=Serin, B=Threonin, B=Prolin, D=Valin, E=Glycin, F=Alanin, G= γ -Aminobuttersäure und α -Aminobuttersäure, H=Isoleucin (H_2 , oberer Teil dieser Reihe von Flecken) und Phenylalanin (H_1 , unterer Teil dieser Reihe von Flecken) (Identifizierung der einzelnen Aminosäuren wurde durch weitere Untersuchungen durchgeführt.)

Wie aus dem Chromatogramm in Abb. 2 entnommen werden kann, trotz der obenberührten Schwankungen der R_f -Werte, wenn man die Aminosäuren in Fraktion 1, 3 und 4 feststellt, so werden schon alle neutrale Aminosäuren in der originalen Fraktion IV klar, ohne diejenigen in Fraktion 2 und 5 nachzuweisen. So wurden Fraktion 1, 3 und 4 auf Filtrierpapier aufgebracht, mit Butanol /Essigsäure entwickelt, und nach teilweise gefärbten Flecken 1. Fraktion wurde in 4 Stücken (A-D), 3. Fraktion in 5 Stücken (D, E, F, H_1 und H_2) und 4. Fraktion in 7 Stücken (D, E, F, G_1 , G_2 , H_1 und H_2) geteilt und mit Wasser extrahiert. Die so isolierten Substanzen wurden unter Verwendung von verschiedenen Lösungsmitteln papierchromatographisch mit den reinen Aminosäuren identifiziert, wie die Resultate in Tabelle II zeigen. Auf diese Weise wurden in Fraktion IV Serin, Threonin, Alanin, Prolin, Glycin, Valin, Phenylalanin, Isoleucin, α -Amino- und γ -Aminobuttersäuren nachgewiesen.

Von den Resultaten sind folgende bemerkenswert: Obwohl keine quantitative Bestimmung der Aminosäuren durchgeführt wurde, konnte aus der Färbung der Papierchromatogramme geschätzt werden, dass Histidin reichlich aber Arginin nicht viel vorhanden war.

Leucin und Methionin wurden nicht nachgewiesen (wie es aus Tabelle II ersichtlich ist), obwohl sie in dem Hydrolysate der Zahn-

TABLE II

*R_f-Werte der isolierten Substanzen und der massgebenden
reinen Aminosäuren mit verschiedenen Lösungsmitteln*

Unterfraktionen	Bu/Ac	Py/Bu	Py	Phen	Et
1A	0.14	0.24	0.54	0.35	0.01
Serin	0.14	0.24	0.55	0.35	0.01
1B	0.18	0.33	0.65	0.49	
Threonin	0.19	0.32	0.64	0.49	
3F & 4F	0.22	0.31	0.60	0.57	0.04
Alanin	0.22	0.31	0.61	0.57	0.04
1C	0.35	0.26	0.68	0.85	
Prolin	0.36	0.27	0.68	0.84	
3E & 4E	0.15	0.14	0.48	0.40	0.48
Glycin	0.16	0.13	0.48	0.40	0.47
1D & 3D & 4D	0.40	0.50	0.75	0.76	0.17
Valin	0.40	0.51	0.74	0.76	0.18
(Methionin)	0.35	0.42	0.73	0.80	
3H ₁ & 4H ₁	0.52	0.55	0.75	0.52	0.25
Phenylalanin	0.52	0.54	0.74	0.53	0.25
3H ₂ & 4H ₂	0.55	0.51	0.71	0.80	0.41
Isoleucin	0.55	0.51	0.70	0.80	0.42
(Ieucin)	0.56	0.55	0.79	0.81	
4G ₂	0.31	0.39	0.70	0.69	0.25
α -Aminobuttersäure	0.31	0.39	0.70	0.69	0.25
4G ₁	0.31	0.16	0.54	0.78	0.09
γ -Aminobuttersäure	0.32	0.16	0.55	0.78	0.09

Erklärung: 1A=Die von der 1. Fraktion isolierte Substanz A.

pulpen vorhanden waren. Dasselbe gilt auch von Hydroxyprolin, welches schon von Saito (3) in Rinderzahnwurzelhaut und von Hess (4) in Dentin sogar 13.10%ig gefunden wurde.

Es ist auch bemerkenswert, dass freie α -Aminobuttersäure in Zahnpulpe gefunden wurde, da sie bisher nur beschränkte Verbreitung in der Natur zeigte. So wurde sie in den Wurzelknoten der Leguminosen, in Schwamm und in Harn von viel Methionin verabreichten Menschen gefunden. Work (5, 6) isolierte sie aus *Corynebacterium diphtheriae*, Meisenheimer (7) aus Hefe und Oikawa (8) aus Kammerwasser von Walfischau-

gen. γ -Aminobuttersäure ist auch keine gewöhnliche Aminosäure, die den Eiweisskörper ausmacht. Sie wurde bisher in Magensaft, Speichel, Säugetiergehirn, Hundesitzbeinnerven (9) und Walfischpancreas (10) gefunden. In Pflanzenreich wurde sie in Wurzelknoten der Leguminosen, in Apfel und in Kelch von "Kaki"-Frucht gefunden.

ZUSAMMENFASSUNG

In dem wässrigen Extrakt von Rinderzahnpulpen wurden folgende freie Aminosäuren nachgewiesen: Lysin, Arginin, Histidin, Glutamin-, und Asparaginsäuren, Serin, Thre-

onin, Prolin, Alanin, Valin, Phenylalanin, Isoleucin, α - und γ -Aminobuttersäuren.

Herrn Prof. Dr. M. Tomita spreche ich für seine freundliche Anleitung meinen verbindlichsten Dank aus.

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Ueber die Freien Aminosaeuren in Zahnhartgewebe

Von SEIICHI MOTOMURA

(Aus dem Biochemischen Laboratorium der Pharmazeutischen
Frauen-Hochschule zu Kobe, Kobe*)

(Der Schriftleitung zugegangen am 23. Januar 1961)

Die organischen Substanzen in Zahnhartgewebe betragen nach Le Fevre und Manly (1) 1.7% in Schmelz und 17.5% in Dentin. Nach Araya (2) bestehen die in Zahnschmelz vorkommenden Eiweissstoffe

mensetzungen der in Zahnschmelz und Dentin vorkommenden unlöslichen Eiweissstoffe bestimmt.

In Bezug auf die löslichen Eiweisstoffe, gibt es nur wenige Mitteilungen. Stack (4)

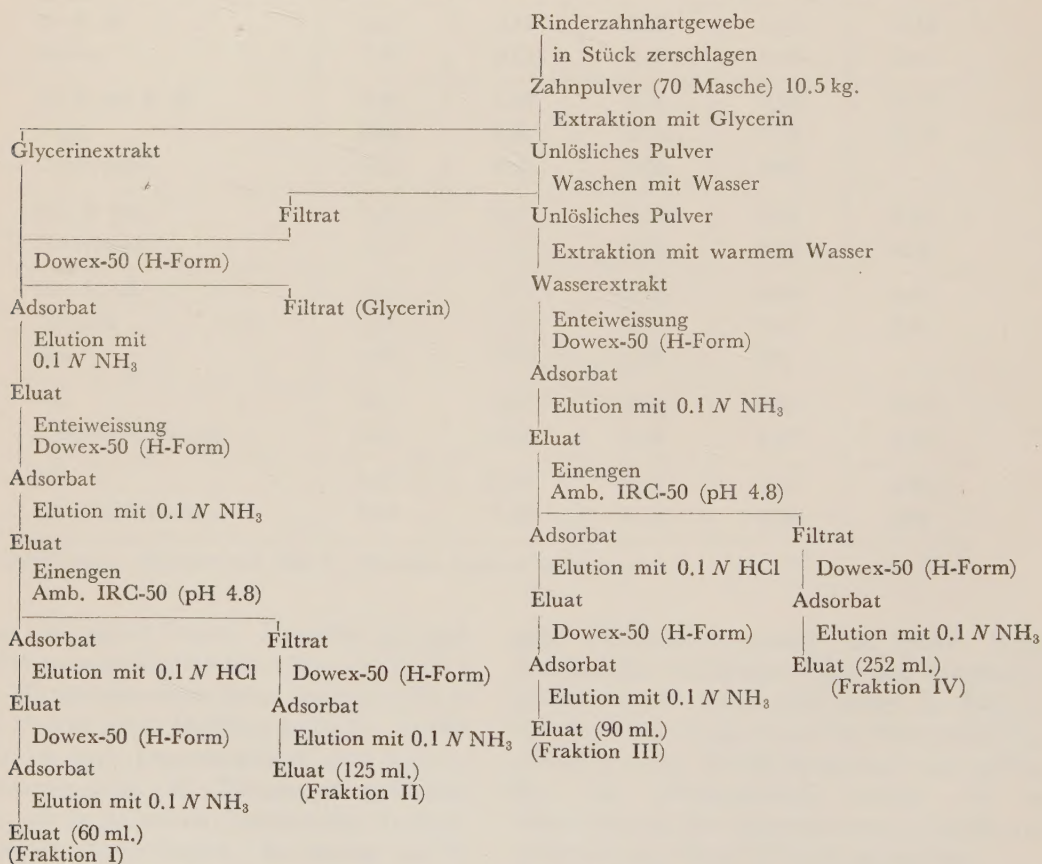


Abb. 1. Fraktionierungsverfahren der Extrakte von Zahnhartgewebe.

grösstenteils aus Keratin, während die in Dentin, aus Kollagen bestehen. Hess, Lee und andere (3) haben die Aminosäurezusam-

wies in Zahnschmelz 0.17% von löslichen Eiweissstoffen und 0.157% von Peptiden nach, während Karshan (5) 0.3, 0.065 bzw. 0.02% von Eiweissstoffen in drei nacheinander folgenden Wasserextrakten des pulverisierten

* Direktor: Prof. Dr. M. Tomita.

Dentins nachwies.

Der Verfasser hat die freien Aminosäuren in dem Zahnhartgewebe untersucht, um sie mit den von ihm* in Zahnpulpen bzw. *Nervus mandibularis* von Rinder nachgewiesenen freien Aminosäuren zu vergleichen.

METHODIK

Material—Frische Hartgewebe der Rinderzähne wurde zerschmettert. 10,5 kg. von 70 Masche Zahnhartgewebepulver wurden in Glycerin 33 Monate lang eingetaucht. Das unlösliche Pulver wurde vom Glycerin abgetrennt und mit Wasser gründlich gewaschen. Das Washwasser wurde mit dem Glycerinextrakt vereinigt und als Glycerinextrakt weiter verarbeitet. Das gewaschene Pulver wurde mit warmem Wasser 4mal extrahiert und der Wasserextrakt wurde von dem Glycerinextrakt getrennt behandelt.

Das Glycerinextrakt wurde mit Wasser 20 mal verdünnt und in eine mit Dowex-50 (H-Form) gefüllte Säule übergeführt. Nach vollständiger Ausspülung mit Wasser, wurde das Adsorbat mit 0,1 N NH_3 eluiert. Das Eluat wurde noch einmal mit Dowex-50 gleicher Weise behandelt. Dann wurde es mit Amberlite IRC-50 bei pH 4,8 in zwei Fraktionen, I und II, fraktioniert. Fraktion I bestand aus basischen Aminosäuren und Fraktion II aus neutralen und sauren.

Das Wasserextrakt wurde wie bei Glycerinextrakt behandelt: Zuerst wurde es durch Tannin-Bleimethode enteiweißt, mit Dowex-50 gereinigt und mit Amberlite IRC-50 in zwei Fraktionen, III und IV, fraktioniert. Davon bestand die erstere aus basischen und die letztere aus neutralen und sauren Aminosäuren. Das Fraktionierungsverfahren der beiden Extrakte ist schematisch in Abb. 1 gezeigt.

Säulen- und Papierchromatographie—Die chromatographische Methodik ist dieselbe wie früher geschrieben wurde.** Säulen von Ionenaustauscher, Dowex-50 und von Amberlite IRC-50 wurden benutzt. Fraktionierungen in mehrere Unterfraktionen wurden mit dem automatischen Fraktionsammler durchgeführt.

Lösungsmittel für Papierchromatographie—n-Butanol: Essigsäure: Wasser (Abkürzung, Bu/Ac) 80:20:20, Pyridin: Butanol: Wasser (Py/Bu) 80:20:20, Pyridin: Wasser (Py) 65:35, Phenol (Phen), gesättigt mit einer wässrigen Lösung von 6,3%igem Natriumcitrat und 3,8%igem saurem K-Phosphat.

Papier, Toyo Nr. 50 und 51.

Nachweis von Aminosäuren durch 0,2%ige Ninhydrinlösung in Butanol. Arginin durch Sakaguchi'sche und Histidin durch Pauly'sche Reaktion.

ERGEBNISSE UND BESPRECHUNGEN

Basische Aminosäuren—Fraktion I von Glycerinextrakt und Fraktion III von Wasserextrakt wurden papierchromatographisch untersucht. Wie aus Tabelle I ersichtlich ist, zeigten sie je drei Flecke von Aminosäuren, die mit Lysin, Arginin und Histidin identifiziert werden konnten.

Neutrale und saure Aminosäuren—A. **Glycerinextrakt**: Fraktion II von Glycerinextrakt wurde durch eine Säule von Amberlite IRC-50 in 61 Unterfraktionen fraktioniert. Wie das Papierchromatogramm in Abb. 2 zeigt, war die Trennung der einzelnen Aminosäuren nicht scharf. Daher wurden die Unterfraktionen wieder teilweise in fünf Fraktionen vereinigt, und zwar Fraktion 1 (von Unterfraktion 1 bis 9), Fraktion 2 (von 10 bis 25), Fraktion 3

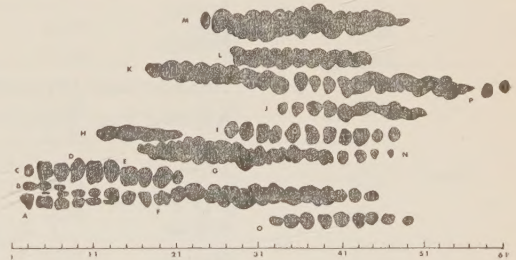


ABB. 2. Chromatogramm der Unterfraktionen von Fraktion II.

A=Asparaginsäure	J= γ -Aminobuttersäure
B=Hydroxyprolin	K=Valin
C=Glutaminsäure	L=Phenylalanin
D=Threonin	M=Leucin
E=Serin	N= β -Alanin und Unbekannte Substanz A
F=Glycin	O=Cystin
G=Alanin	P=Unbekannte Substanz B
H=Prolin	
I=Tyrosin	

(Identifizierung der einzelnen Aminosäuren wurde durch weitere Untersuchungen durchgeführt.)

(von 26 bis 32), Fraktion 4 (von 33 bis 40) und Fraktion 5 (von 41 bis 61). Da alle neutrale und saure Aminosäuren in dem Glycerinextrakt durch Analyse von Fraktion 1, 2 und 5

* Motomura, S., 32. Generaltagung der Japanischen Biochemischen Gesellschaft, Osaka (1959)

** Motomura, S., 32. Generaltagung der Japanischen Biochemischen Gesellschaft, Osaka (1959)

TABELLE I

R_f-Werte der Adsorbate an Amberlite IRC-50 von Glycerin- und Wasserextrakt

Bu/Ac	Adsorbat vom Glycerinextrakt	0.20	0.26	0.37
	Adsorbat vom Wasserextrakt	0.21	0.25	0.37
	Lysin (Hydroxylysin)	0.21		0.20
	Arginin		0.25	
	Histidin			0.38
Py/Bu	Adsorbat vom Glycerinextrakt	0.05		0.11
	Adsorbat vom Wasserextrakt	0.05		0.11
	Lysin (Hydroxylysin)	0.05		0.04
	Arginin		0.05	
	Histidin			0.11
Py	Adsorbat vom Glycerinextrakt	0.31	0.37	0.50
	Adsorbat vom Wasserextrakt	0.31	0.38	0.51
	Lysin (Hydroxylysin)	0.32		0.38
	Arginin		0.38	
	Histidin			0.50
Phen	Adsorbat vom Glycerinextrakt	0.59	0.64	0.70
	Adsorbat vom Wasserextrakt	0.58	0.65	0.70
	Lysin (Hydroxylysin)	0.60		0.18
	Arginin		0.65	
	Histidin			0.70

ausgezählt werden können, wurden nur diese papierchromatographisch mit Butanol-Essigsäure entwickelt. Die Chromatogramme von ihnen wurden mit Ninhydrin teilweise gefärbt, um einzelne Substanzen zu isolieren. Auf diese Weise wurden drei Fraktionen (A-C) aus Fraktion 1, sechs Fraktionen (A-F) aus Fraktion 2 und neun Fraktionen (A-I) aus Fraktion 5 gewonnen.

B. Wasserextrakt: Fraktion IV von Wasserextrakt wurde genau so wie Fraktion II von Glycerinextrakt behandelt und durch eine Säule von Amberlite IRC-50 in 37 Unterfraktionen fraktioniert. Abb. 3 zeigt das Papierchromatogramm von den Unterfraktionen. Um einzelne Aminosäuren papierchromato-

graphisch zu isolieren, wurden sie wieder teilweise in drei Fraktionen vereinigt: Fraktion 1 (von Unterfraktion 1 bis 7), Fraktion 2 (von 8 bis 14) und Fraktion 3 (von 15 bis 37).

Fraktion 3 wurde wieder in 31 Unterfraktionen fraktioniert, um die Bestandteile genauer zu untersuchen, davon das Papierchromatogramm in Abb. 4 gezeigt ist. Dann wurden diese 31 Unterfraktionen wieder in vier Fraktionen vereinigt, nämlich Fraktion 3-1 (von Unterfraktion 1 bis 5), Fraktion 3-2 (von 6 bis 13), Fraktion 3-3 (von 14 bis 24) und Fraktion 3-4 (von 25 bis 31). Fraktion 1, 2 und 3-3 wurden mit Butanol-Essigsäure papierchromatographisch entwickelt, nach

TABELLE II

R_F-Werte der isolierten Substanzen und der massgebenden reinen Aminosäuren mit verschiedenen Lösungsmitteln

	Bu/Ac		Py/Bu		Py		Phen	
G. 1A & 1A	0.12	0.16	0.04	0.15	0.45	0.48	0.16	0.41
Asparaginsäure	0.12		0.04		0.45		0.15	
Glycin		0.16		0.14		0.48		0.40
G. 1B	0.30		0.23		0.65		0.65	
Hydroxyprolin	0.30		0.24		0.66		0.65	
G. 1C & W. 1B	0.17		0.15		0.52		0.27	
Glutaminsäure	0.18		0.15		0.53		0.26	
G. 2A, G. 5B, W. 2A & W. 3-3A	0.16		0.14		0.48		0.40	
Glycin	0.16		0.14		0.48		0.40	
G. 2B & W. 1B	.14 .18 .19		.23 .15 .32		.55 .53 .65		.35 .27 .49	
Serin	.14		.24		.55		.35	
Glutaminsäure	.18		.15		.53		.26	
Threonin	.19		.33		.65		.49	
G. 2C & W. 2C	0.21		0.29		0.61		0.59	
Alanin	0.22		0.30		0.62		0.58	
G. 2D & W. 1C	0.36		0.25		0.68		0.85	
Prolin	0.36		0.26		0.68		0.85	
G. 2E W. 2F	0.40		0.49		0.74		0.76	
Valin	0.40		0.50		0.75		0.76	
G. 2F, G. 5I, W. 2G & W. 3-3G	0.55		0.54		0.79		0.81	
Leucin	0.56		0.55		0.79		0.81	
G. 5A	0.04		0.07		0.46		0.24	
Cystin	0.04		0.08		0.45		0.25	
G. 5C (Unbekannte Substanz A)	0.21		0.12		0.62		0.75	
G. 5D & W. 3-3B	0.24		0.15		0.59		0.66	
β -Alanin	0.23		0.15		0.59		0.66	
G. 5E & W. 2E	0.29		0.57		0.79		0.59	
Tyrosin	0.29		0.58		0.80		0.60	
G. 5F & W. 3-3D	0.31		0.16		0.55		0.78	
γ -Aminobuttersäure	0.32		0.16		0.55		0.78	
G.5G & W.3-3E (Unbekannte Sub. B)	0.40		0.13		0.60		0.80	
G. 5H & W. 3-3F	0.51		0.55		0.75		0.82	
Phenylalanin	0.52		0.55		0.75		0.82	

Abkürzungen: G. 1A=Die aus Fraktion 1 des Glycerinextraktes isolierte Substanz A.
W. 2B=Die aus Fraktion 2 des Wasserextraktes isolierte Substanz B.
usw.

teilweise gefärbten Flecken in entsprechende Stück geteilt und mit Wasser extrahiert. Auf diese wurden drei Substanzen (A-C) aus

menfassend wurden in Glycerinextrakt Asparaginsäure, Glycin, Hydroxyprolin, Glutaminsäure, Serin, Threonin, Alanin, Prolin, Valin,



ABB. 3. Chromatogramm der Unterfraktionen von Fraktion IV.
a=Asparaginsäure h=Tyrosin
b=Glutaminsäure i=Valin
c=Threonin l= β -Alanin
d=Serin m= γ -Aminobuttersäure
e=Glycin n=Unbekannte Substanz B
f=Alanin k=Leucin
g=Prolin w=Phenylalanin
(Identifizierung der einzelnen Aminosäuren wurde durch weitere Untersuchungen durchgeführt.)

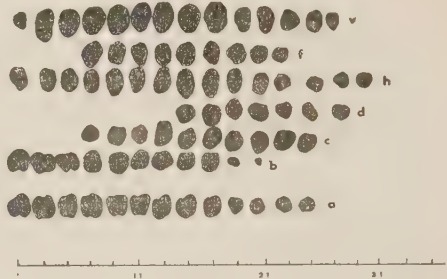


ABB. 4. Chromatogramm der Unterfraktionen von Fraktion 3 des Wasserextraktes.
a=Glycin h=Valin (1-13)
b= β -Alanin Unbekannte Substanz B (14-31)
c=Tyrosin
d= γ -Aminobuttersäure f=Phenylalanin
v=Leucin
(Identifizierung der einzelnen Aminosäuren wurde durch weitere Untersuchungen durchgeführt.)

Fraktion 1, sieben Substanzen (A-G) aus Fraktion 2 und sieben Substanzen (A-G) aus Fraktion 3-3 gewonnen.
Die auf diese Weise von Glycerinextrakt und von Wasserextrakt gewonnenen Fraktionen wurden papierchromatographisch mit den reinen Aminosäuren identifiziert. Wie aus Tabelle II ersichtlich ist, bestanden sie meistens aus einzigen Aminosäuren. Zusammen-

Leucin, Cystin, β -Alanin, Tyrosin, γ -Aminobuttersäure und Phenylalanin nachgewiesen. In Wasserextrakt wurden aber kein Hydroxyprolin noch Cystin gefunden.
Bemerkenswert ist, dass zwei Substanzen gefunden wurden, die mit keinen bekannten Aminosäuren identifiziert wurden konnten. Eine von ihnen wurden von Glycerin- sowie von Wasserextrakt isoliert, während die andere

TABELLE III

R_f-Werte der unbekannten Substanzen sowie der massgebenden reinen Aminosäuren mit verschiedenen Lösungsmitteln

	Bu/Ac	Py/Bu	Py	Phen
Unbekannte Substanz B (P)	0.40	0.13	0.60	0.80
Valin (P)	0.40	0.50	0.75	0.76
Methionin (P)	0.35	0.42	0.73	0.80
Tryptophan (P)	0.38	0.49	0.72	0.75
γ -Amino- β -hydroxy-buttersäure (P)	0.24	0.16	0.59	0.54
β -Amino-isobuttersäure (P)	0.38	0.22	0.65	0.74
Unbekannte Substanz A (G)	0.21	0.12	0.62	0.75
γ -Aminocrotonsäure (G)	0.31	0.08	0.52	0.60

G=Gelber Fleck P=Purpurfarbiger Fleck

nur von Glycerinextrakt isoliert wurde. Aber sie konnten leider nicht kristallisiert werden, wegen der zu wenigen Menge. Da die ω -Aminosäuren durch Amberlite IRC-50 säulenchromatographisch gewöhnlich langsamer eluiert wurden, könnten die beiden unbekannten Substanzen solch sein. Aber wie in Tabelle III gezeigt ist, liess die eine unbekannte Substanz, A, die wie γ -Aminocrotonsäure gelblichen Fleck gab, sich mit dieser nicht identifizieren. Die andere unbekannte Substanz, B, konnte weder mit Valin, Methionin, Tryptophan, γ -Amino- β -hydroxybuttersäure noch β -Amino-isobuttersäure identifiziert werden.

Zum Schluss sei über den Vergleich von den Zusammensetzungen der freien Aminosäuren von Zahnpulpe, *Nervus mandibularis* und Zahnhartgewebe einiges erwähnt. Aus diesen drei Geweben wurden insgesamt Glutaminsäure, Asparaginsäure, Lysin, Arginin, Histidin, Serin, Cystin, Glycin, Threonin, Prolin, Hydroxyprolin, Valin, Leucin, Isoleucin, Phenylalanin, Alanin, Tyrosin, α -Aminobuttersäure, β -Alanin, γ -Aminobuttersäure und zwei unbekannte Substanzen isoliert. Davon fehlte Serin in *Nervus mandibularis*, während Hydroxyprolin, Cystin und Tyrosin nur in Zahnhartgewebe gefunden wurden. Isoleucin und α -Aminobuttersäure wurden nur in Zahnpulpe gefunden, während Leucin in ihr fehlte.

ZUSAMMENFASSUNG

In dem Zahnhartgewebe von Rind wurden folgende freie Aminosäuren mit Glycerin extrahiert: Glutaminsäure, Asparaginsäure, Lysin, Arginin, Histidin, Serin, Threonin, Cystin, Prolin, Hydroxyprolin, Tyrosin, Valin, Phenylalanin, Leucin, β -Alanin und γ -Aminobuttersäure. Mit Wasser wurde weder Cystin noch Hydroxyprolin extrahiert.

Aus Zahnhartgewebe wurden zwei unbekannte Substanzen isoliert, welche ω -Aminosäuren zu sein scheinen. Die eine gab gelben Fleck durch Ninhydrin aber konnte mit γ -Aminocrotonsäure, die auch gelben Fleck aufzeigt, nicht identifiziert werden, während die andere nicht mit β -Amino-isobuttersäure identifiziert wurde.

Herrn Prof. Dr. M. Tomita spreche ich für seine freundliche Anleitung bei der Ausführung dieser Arbeit meinen verbindlichsten Dank aus.

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Ueber die Freien Aminosäuren in *Nervus Mandibularis*

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(Der Schriftleitung zugegangen am 23. Januar 1961)

Nervus mandibularis ist der dritte Ast des Trigemini, des 5. Gehirnnerven. Hinsichtlich der biochemischen Zusammensetzung des Nerven, ausser Wasser, Salzen und Eiweisskörpern, wurden Nukleoproteide, Lecithin, Cholesterin, Cerebroside, Glycogen, Acetylcholin und andere Stoffe sowie verschiedene Enzyme nachgewiesen. Prof. Masaji Tomita (1) hat in seinem Werk, "Vergleichende Biochemie", als anorganische Elemente in peripherischen Nerven von K und Mn bei Kaninchen, Ca bei Hund, und K und Mn bei Katz erwähnt. Er hat dabei die Aminosäurezusammensetzungen der Grosshirn der verschiedenen Tiere angestellt. S. Moore und W. H. Stein (2), sowie J. H. Quastel und seine Mitarbeiter haben in 1956 die freien Aminosäuren im Gehirn quantitativ bestimmt. In bezug auf die Aminosäuren in peripherischen Nerven hat in 1951 R. M. May (4) γ -Aminobuttersäure in Sitzbeinnerven von Hund nachgewiesen.

Der Verfasser hat in vorliegenden Versuchen die freien Aminosäuren in *Nervus mandibularis* von Rind untersucht.

METHODIK

Material—Aus 397 Rindern wurden 11.7 kg. von *Nervus mandibularis* frisch herausgenommen und nach Zerkleinerung und Zerreibung mit Alkohol 17 Monate lang übergossen. Dann wurde das Mazerat von dem Alkoholextrakt abgetrennt und mit warmem Wasser 4 mal extrahiert. Alkohol- und Wassereextrakt wurden vereinigt und unter vermindertem Druck von Alkohol befreit. Der extrakt wurde, nach der Entfernung vom Fett mit Äther, durch Tannin-Blei-Methode enteiwisst.

Säulen- und Papierchromatographie—Die chromatographische Methodik ist dieselbe wie in der vorigen

Arbeit*. Säulen von Ionenaustauscher, Dowex-50 (H-Form) und Amberlite IRC-50 wurden gebraucht.

Lösungsmittel für Papierchromatographie waren, *n*-Butanol: Essigsäure: Wasser (Abkürzung, Bu/Ac) 80:20:20, Pyridin: Butanol: Wasser (Py/Bu) 80:20:20, Pyridin: Wasser (Py) 65:35, Phenol (Phen), gesättigt mit einer wässrigen Lösung von 6.3%igem Na-Citrat und 3.7%igem saurem K-Phosphat.

Papier, Toyo Nr. 50 und 51.

Nachweis von Aminosäuren durch 0.2%ige Ninhydrinlösung in Butanol. Arginin durch Sakaguchi'sche und Histidin durch Pauly'sche Reaktion.

ERGEBNISSE DER VERSUCHE

Fraktionierung und Isolierung der freien Aminosäuren—Die freien Aminosäuren in dem enteiwissten Extrakt wurden mit Dowex-50 adsorbiert und mit 0.1 *N* NH₃ eluiert. Das unter vermindertem Druck eingegangte Eluat wurde mit Amberlite IRC-50 säulenchromatographisch nach dem in Abb. 1 gezeigten Verfahren in zwei Fraktionen I und II, nämlich die von basischen und die von neutralen und sauren Aminosäuren fraktioniert.

Basische Aminosäuren—Zu der Fraktion I (von basischen Aminosäuren) wurde Pikrinsäure hinzugesetzt, um 100 mg. von gelblichen, nadelförmigen und seidenglänzenden Kristallen zu gewinnen. Die Kristalle schmelzen bei 253°C unter Zersetzung, und die Analysenzahlen stimmten mit denjenigen, die für Lysin-pikrat berechnet wurden.

Analysenzahlen der Kristalle:

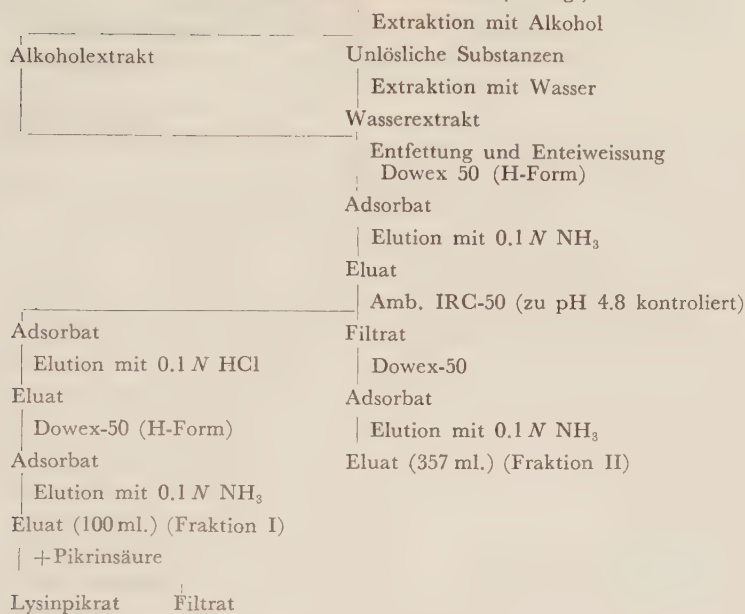
Gef.	C 38.55	H 4.74	N 18.61
Ber.	38.40	4.59	18.66

Berechnet nach der Bruttoformel, C₆H₁₄-O₂N₂·C₆H₃O₉N₃ von Lysin-pikrat.

Die Mutterlauge von Lysin-pikrat wurde

* Direktor: Prof. Dr. M. Tomita.

* Motomura, S., 32. Generaltagung der Japanischen Biochemischen Gesellschaft, Osaka (1959)

Nervus mandibularis von Rinder (11.7 kg.)ABB. 1. Fraktionierungsverfahren der Extrakte von *Nervus mandibularis*.

von Pikrinsäure befreit und papierchromatographisch mit verschiedenen Lösungsmitteln

TABELLE I

R_f-Werte der Versuchslösung und der massgebenden reinen Aminosäuren

Versuchslösung		0.21	0.24	0.37
Bu/Ac	Lysin	0.21		
	Arginin		0.25	
	Histidin			0.38
Versuchslösung		0.06		0.10
Py/Bu	Lysin	0.05		
	Arginin		0.05	
	Histidin			0.11
Versuchslösung		0.31	0.38	0.51
Py	Lysin	0.32		
	Arginin		0.38	
	Histidin			0.51
Versuchslösung		0.59	0.64	0.69
Phen	Lysin	0.60		
	Arginin		0.65	
	Histidin			0.70

untersucht. Wie in Tabelle I gezeigt ist, wurden die Aminosäureflecke mit Lysin, Arginin und Histidin identifiziert.

Neutrale und saure Aminosäuren—Da fraktion II mehrere neutrale und saure Aminosäuren enthielt, wurde sie mit Dowex-50 säulenchro-

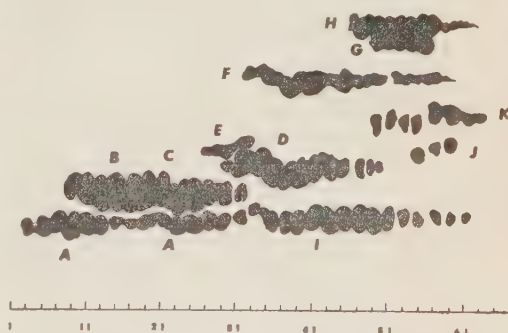


ABB. 2. Chromatogramm der Unterfraktionierung von Fraktion II.

A = Asparaginsäure	G = Phenylalanin
B = Glutaminsäure	H = Leucin
C = Threonin	I = Glycin
D = Alanin	J = β -Alanin
E = Prolin	K = γ -Aminobuttersäure
F = Valin	

Über die Identifizierung der einzelnen Aminosäuren siehe Text.

matographisch in 65 Unterfraktionen fraktioniert. Aber die Trennung der einzelnen Aminosäuren war nicht scharf, ausser einigen ersten Fraktionen, wie das Chromatogramm in Abb. 2 zeigt. Daher wurden die Unterfraktionen wieder in sieben Fraktionen vereinigt, um papierchromatographisch einzelne Aminosäuren zu identifizieren; Fraktion 1 (von Unterfraktion 1 bis 5), Fraktion 2 (von 6 bis 13), Fraktion 3 (von 14 bis 26), Fraktion 4 (von 27 bis 34), Fraktion 5 (von 35 bis 47), Fraktion 6 (von 48 bis 60) und Fraktion 7 (von 61 bis 65).

Wie aus Abb. 2 ersichtlich ist, wenn diejenigen Aminosäuren, die in Fraktion 1, 2, 3, 5 und 6 vorhanden sind, geklärt werden, so

TABELLE II

R_f-Werte der 2., 3., und 5. Fraktionen sowie der massgebenden reinen Aminosäuren

Bu/Ac	2. Fraktion	0.11	0.19		
	3. Fraktion	0.12	0.19	0.35	
	Asparaginsäure	0.12			
	Glutaminsäure		0.18		
	Threonin			0.19	
	Prolin				0.36
Py/Bu	2. Fraktion	0.03	0.14	0.25	
	3. Fraktion	0.03	0.15	0.26	0.32
	Asparaginsäure	0.04			
	Glutaminsäure		0.15		
	Threonin			0.26	
	Prolin				0.33
Py	2. Fraktion	0.45	0.52	0.64	
	3. Fraktion	0.45	0.53	0.65	0.68
	Asparaginsäure	0.45			
	Glutaminsäure		0.53		
	Threonin			0.65	
	Prolin				0.68
Phen	2. Fraktion	0.15	0.25	0.48	
	3. Fraktion	0.15	0.26	0.48	0.84
	Asparaginsäure	0.15			
	Glutaminsäure		0.26		
	Threonin			0.49	
	Prolin				0.85

werden ohne weiteres alle Aminosäuren, die in Fraktion II enthalten sind, folglich alle saure und neutrale Aminosäuren in Original-extrakt, klar.

In Fraktion 1 konnte nur Asparaginsäure nachgewiesen werden. Von Fraktion 2, 3 und 5 wurden die Resultate in Tabelle II, gezeigt: Fraktion 2 zeigte manchmal drei Flecke, welche mit Asparagin-, Glutaminsäure, und Threonin identifiziert wurden. Fraktion 3 zeigte vier Flecke, die mit den oben genannten Aminosäuren und Prolin identifiziert wurden. Fraktion 5 zeigte vier Flecke, die mit Glycin, Alanin, Valin und Leucin identifiziert wurden.

In Fraktion 6 wurde das Vorhandensein von β -Alanin und γ -Aminobuttersäure geschlossen. Daher wurde sie wieder säulenchromatographisch mit Dowex-50 in 50 Unter-

Bu/Ac	5. Fraktion	0.15	0.21	0.40	0.52	0.56
	Glycin	0.16				
	Alanin		0.22			
	Valin			0.40		
	Phenylalanin				0.52	
	Leucin					0.56
Py/Bu	5. Fraktion	0.14	0.29	0.49	0.55	0.54
	Glycin	0.14				
	Alanin		0.30			
	Valin			0.50		
	Phenylalanin				0.55	
	Leucin					0.55
Py	5. Fraktion	0.46	0.61	0.74	0.76	0.79
	Glycin	0.46				
	Alanin		0.61			
	Valin			0.75		
	Phenylalanin				0.76	
	Leucin					0.79
Phen	5. Fraktion	0.40	0.58	0.76	0.82	0.80
	Glycin	0.40				
	Alanin		0.58			
	Valin			0.76		
	Phenylalanin				0.82	
	Leucin					0.81

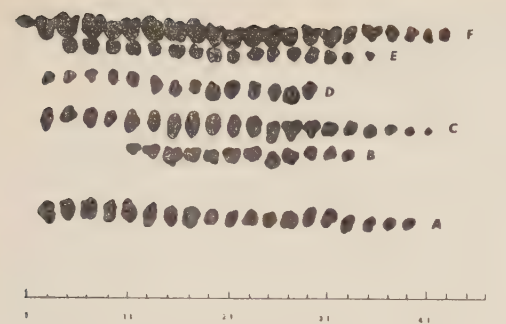


ABB. 3. Chromatogramm der Unterfraktionen von Fraktion 6.
A=Glycin D=Valin
B= β -Alanin E=Phenylalanin
C= γ -Aminobuttersäure F=Leucin
(Identifizierung der einzelnen Aminosäuren wurde durch weitere Untersuchungen durchgeführt.)

fraktionen fraktioniert, um die beiden Aminosäuren zu isolieren.

Wie aus dem Papierchromatogramm in Abb. 3 ersichtlich, war die Trennung der einzelnen Aminosäuren nicht scharf. So wurden die Unterfraktionen wieder teilweise vereinigt: Fraktion 6-1 (von Unterfraktion 1 bis 10), Fraktion 6-2 (von 11 bis 36) und Fraktion 6-3 (von 37 bis 50). Dann wurde Fraktion 6-2 papierchromatographisch weiter fraktioniert und zwar nach den R_f -Werten

TABELLE III

R_f -Werte der Unterfraktionen B und C sowie der massgebenden reinen Aminosäuren

	Bu/Ac	Py/Bu	Py	Phen
Unterfraktion β -Alanin	0.24 0.23	0.15 0.15	0.58 0.58	0.66 0.68
Unterfraktion γ -Aminobuttersäure	0.31 0.32	0.16 0.16	0.55 0.55	0.78 0.78

in sechs Unterfraktionen, A, B, C, D, E und F. In den sechs Unterfraktionen wurden papierchromatographisch je eine Aminosäure nachgewiesen und zwar in A Glycin, in B β -Alanin, in C γ -Aminobuttersäure, in D Valin, in E Phenylalanin und in F Leucin, davon die Resultate von Unterfraktion B und C in Tabelle III wiedergegeben sind.

Besprechung der Ergebnisse—Einige Besonderheiten wurden in der Zusammensetzung der freien Aminosäuren in *Nervus mandibularis* von Rind in Vergleich mit denen des Grosshirns beobachtet. In *Nervus mandibularis* konnten nicht freie Asparagin, Glutamin, β -Amino-Isobuttersäure, Serin, Ornithin, Isoleucin, Tyrosin, Tryptophan, Methionin und Taurin nachgewiesen werden.

ZUSAMMENFASSUNG

In dem *Nervus mandibularis* von Rind wurden chromatographisch folgende freie Aminosäuren nachgewiesen: Lysin, Arginin, Histidin, Asparaginsäure, Glutaminsäure, Threonin, Glycin, Prolin, Leucin, Phenylalanin, Valin und Alanin, sowie zwei ω -Aminosäuren: β -Alanin und γ -Aminobuttersäure.

Herrn Prof. Dr. M. Tomita spreche ich hiermit für seine freundliche Anleitung bei der Ausführung dieser Arbeit meinen verbindlichsten Dank aus.

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Electric Birefringence of Rabbit Tropomyosin

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(Received for publication, January 23, 1961)

Since its discovery by Bailey (1) as the third structural protein in muscle, tropomyosin has been studied from physico-chemical and comparative biochemical standpoints (2-7). In the absence of salts rabbit tropomyosin solutions have very high viscosity, which is greatly and instantaneously decreased by the addition of neutral salts. This decrease of viscosity is interpreted as being due to the dissociation of linearly aggregated tropomyosin particles. On the basis of the above effect of salts, Bailey (8) pointed out that this linear aggregation was caused by electrostatic attraction due to the asymmetric distribution of positive and negative charges on tropomyosin molecule. If this is true, it is naturally expected that the infinite dilution of tropomyosin solutions gives dispersed tropomyosin monomers. Experiments on osmotic pressure and light scattering by several authors (2-9) showed, however, that at ionic strength less than 0.1, the extrapolation to infinite dilution gave a molecular weight much higher than that of monomer, 53,000, which is found in concentrated salt or urea solutions. This contradictory result has made it difficult to analyze the polymerization phenomenon of tropomyosin as a reversible physical equilibrium.

In the present work, it has been undertaken to investigate the polymerization phenomenon of rabbit tropomyosin under various conditions by the electric birefringence method developed by Benoit (10). This method is very useful, because the analysis of the transient birefringence behavior gives us informations on the particle length and the electric property of tropomyosin molecules. Measurements can be made down

to very low concentration of tropomyosin on account of its large electric birefringence.

As the results, it was found that the infinite dilution completely depolymerized the aggregated tropomyosin particles to monomers even under the salt-free solvent conditions, and that tropomyosin molecule had a permanent dipole moment. It has also shown that the polymerization was not always due to the head-to-tail association. The general feature, however, can be qualitatively explained by the scheme of linear polymerization. The binding constant between tropomyosin molecules was estimated on this scheme and the process of polymerization was discussed.

EXPERIMENTAL

Preparation of Tropomyosin—The rabbit tropomyosin was prepared by Bailey's method (11). A crude tropomyosin extracted from ethanol-ether dry muscle in the presence of *M* KCl was precipitated at pH 4.8, and the solution obtained by dissolving the precipitate at pH 7.0 was salted out within 41 to 70% ammonium sulfate saturation. The isoelectric precipitation in the presence of *M* KCl and the subsequent salting-out within a narrower ammonium sulfate saturation range were repeated twice more. The paste obtained was dialyzed against distilled water until the solution became free from ammonium sulfate. The tropomyosin solution prepared in this way contained no appreciable amount of impurities, according to electrophoretic and sedimentational analysis. The concentration of tropomyosin was determined by a differential refractometer, using a value of refractive index increment 0.188 (9).

Apparatus—A rectangular electric pulse applied to the solution in a Kerr cell was given through a transient connection between an electrode of the cell and an out-put of the voltage divider by the use of a vacuum mercury switch. The connecting time could

be controlled from about 10 to 100 millisecc. The applied voltage can be varied potentiometrically from zero to 450 volts by the voltage division of a stabilized high voltage supply. By transient discharge of the charges stored in the electric condensers of high capacity (2,400 microfarads) connected in parallel with the out-put of the voltage divider, a stable pulse can be made even at 150 ohms load without intermittent arcing at the point of contact. The rise and decay time of the pulse was about 0.3 microsec. A reversing pulse generator constructed by electronic units was also employed to measure directly the contribution of permanent dipole to the electric birefringence. The rise and decay time of this reversing pulse was about 2 microsec. Electrodes of the Kerr cell were constructed by two platinum plates, the distance of which was 3.0 mm. A zirconium lamp was used as the light source. A $\lambda/4$ plate was interposed between the Kerr cell and an analysing nicol prism rotated 4° from the crossed position in accordance with the method of O'Konski *et al.* (12). The signal from the photomultiplier was amplified by a synchronized oscilloscope, and the amplified signal on the oscilloscope screen was photographed. The equivalent capacitance distributed in the detecting circuit was about 70 microfarads. The resistance of the photomultiplier anode could be varied from 3 to 250 kilohms.

Calculations of Kerr Coefficient and Length—Optical phase retardation σ is determined by steady state signal intensity ΔI measured on the oscilloscope by the following relation,

$$\Delta I = I \sin \frac{\sigma}{2} \cdot \sin \left(2\theta + \frac{\delta}{2} \right), \quad 1$$

where θ is the angle of the analyzer rotated from the crossed position, and I is the incident light intensity. The Kerr coefficient B and the specific Kerr coefficient B/C is calculated by the following relation,

$$B = \frac{\Delta n}{\lambda E^2} = \frac{\delta}{2\pi l E^2}, \quad 2$$

where C is the protein concentration in g./ml., Δn the degree of birefringence, E the electric field strength in c.g.s. unit, λ the wave length of light, and l is the optical path of the cell.

If the particle is a prolate ellipsoid, the length of the long axis of the particle L is related to the decay time of the transient electric birefringence τ as follows (13),

$$\tau = \frac{\pi \eta_0 L^3}{6KT P^2 r(p)}, \quad 3$$

where η_0 is the viscosity of the solvent, p is the axial ratio of the ellipsoid, and $P^2 r(p)$ is a function of p ,

$$P^2 r(p) = \frac{3}{2} \left(\frac{p^4}{p^4 - 1} \right) \left\{ -1 + \frac{2p^2 - 1}{2p \sqrt{p^2 - 1}} \ln \frac{p + \sqrt{p^2 - 1}}{p - \sqrt{p^2 - 1}} \right\}. \quad 4$$

RESULTS AND ANALYSIS

Typical oscillograms of the electric birefringence under the applied reversing pulse are shown in Fig. 1. The transient decrease of the birefringence due to reversal of the

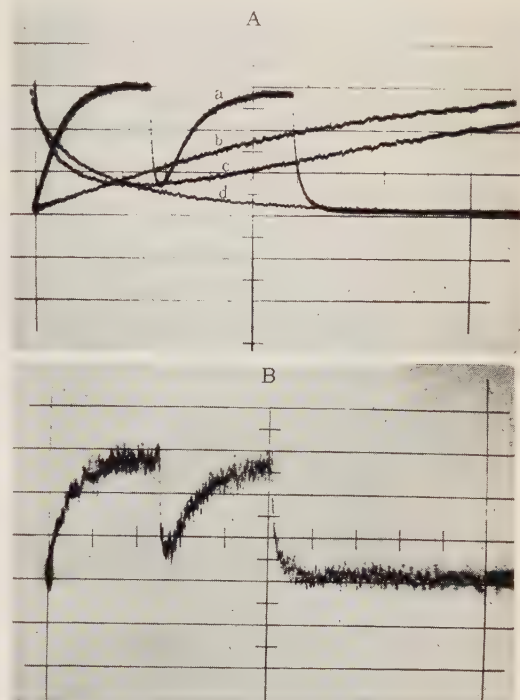


FIG. 1. Oscillograms of the electric birefringence under the applied reversing pulse. A. Protein concentration 2×10^{-3} g./ml., urea 0.45 M, phosphate buffer 2 mM (pH 6.9), applied field strength 90 volt./cm. Sweep speed; 10 millisecc./scale division for curve a, 1 millisecc./scale division for curves b, c, d. B. Protein concentration 2.5×10^{-4} g./ml., urea 0.45 M, phosphate buffer 2 mM, applied field strength 400 volt./cm. Sweep speed; 0.2 millisecc./scale division.

applied electric field indicates directly that tropomyosin has a permanent dipole moment parallel to the long axis of the molecule. By analysis of the curves according to Tinoco's theory (14), it is found that the contribution of permanent dipole to the birefringence is about twice as large as that of induced dipole. Such a value of the ratio of the contribution of two kinds of dipoles is always

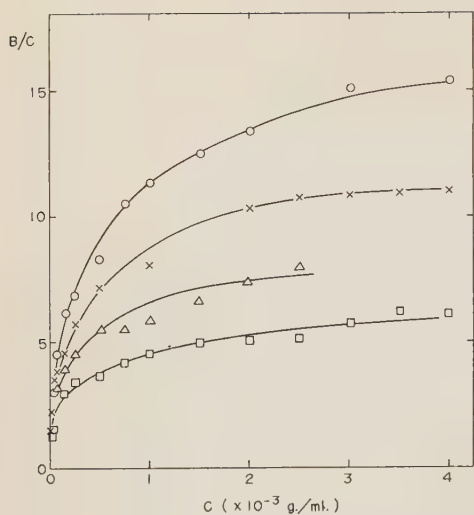


FIG. 2A. The relation between the specific Kerr coefficient (B/C) and the protein concentration (C) at various concentrations of KCl. Phosphate buffer 2 mM (pH 6.9). Temperature 13°C. $\circ$: 0 mM, $\times$: 2 mM, $\triangle$: 6 mM, $\square$: 12 mM.

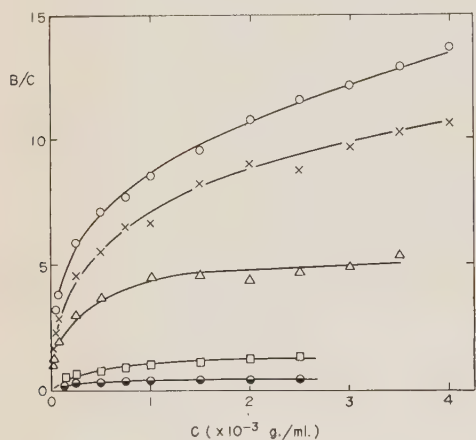


FIG. 2B. The relation between the specific Kerr coefficient (B/C) and the protein concentration (C) at various concentration of urea. Phosphate buffer 2 mM. Temperature 13°C. $\circ$: 0.45 M, $\times$: 0.9 M, $\triangle$: 1.8 M, $\square$: 2.7 M, $\bullet$: 3.6 M.

found in different conditions of solutions. As seen in Fig. 1A, the decay curve of the birefringence is considerably deviated from a simple exponential curve. This fact shows that tropomyosin molecules in solution are aggregated to particles of various lengths.

Depolymerization by Dilution—Figs. 2A and 2B

show the relations between the specific Kerr coefficient and the protein concentration at various concentrations of KCl and urea. The specific Kerr coefficient decreases with the protein concentration. At infinite dilution, it becomes about $0.3 \text{ cm}^4/\text{g. volt}^2$ independently of concentration of salt or urea.

The average length of the aggregated tropomyosin particles can be calculated from the decay time of birefringence by the use of the equation 3, under the assumption that the aggregation is a rigid rod-like. In the present calculation the axial ratio of tropomyosin particles was always assumed to be 25; the value was proposed for tropomyosin monomer (2). This procedure is permissible, because the further increase of axial ratio by the linear aggregation has little influence on the numerical value of the estimated length. The relations between the average particle length and the protein concentration at various concentrations of KCl and urea are shown in Figs. 3A and 3B. The particle length decreases with dilution of the tropomyosin

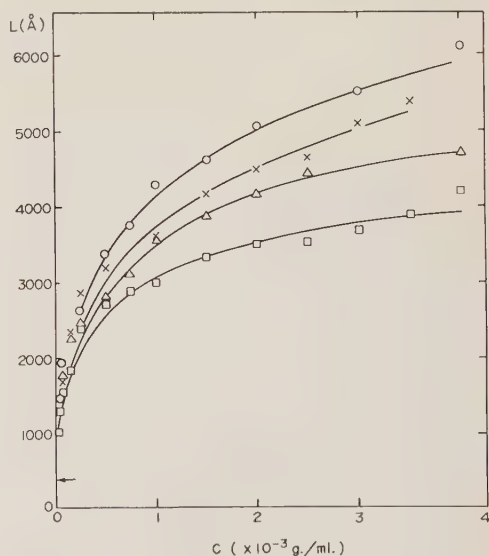


FIG. 3A. The relation between the average particle length (L) and the protein concentration (C) at various concentrations of KCl. The solutions are the same as those in Fig. 2A. $\circ$: 0 mM, $\times$: 2 mM, $\triangle$: 6 mM, $\square$: 12 mM. The extrapolated value to infinite dilution is indicated by the arrow.

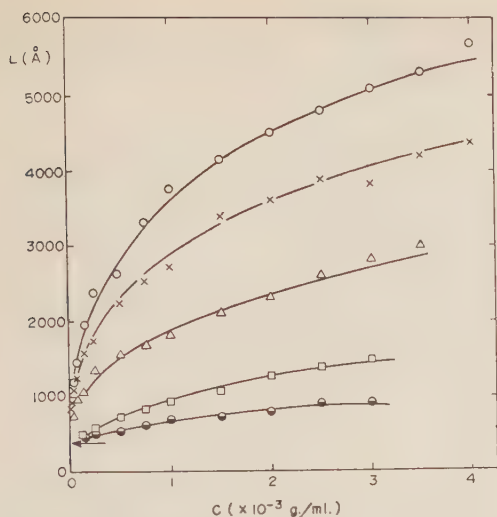


FIG. 3 B. The relation between the average particle length (L) and the protein concentration (C) at various concentrations of urea. The solutions are the same as those in Fig. 2 B. $\circ$: 0.45 M , $\times$: 0.9 M , $\triangle$: 1.8 M , $\square$: 2.7 M , $\bullet$: 3.6 M . The extrapolated value to infinite dilution is indicated by the arrow.

solution. At infinite dilution, the length of tropomyosin tends to 400 ± 50 Å independently of the solvent conditions. This value is in good agreement with the length of tropomyosin monomer observed by Bailey *et al.* (2) in strongly depolymerizing solvents. Therefore, our experiment shows that even under the salt-free condition the aggregated tropomyosins are depolymerized to monomers by infinite dilution. This result can be reasonably expected if the aggregation of tropomyosins is a reversible physical polymerization.

Analysis—To find how tropomyosin molecules are aggregated, the above experimental results are compared with the following calculation. Let us consider of a solution which consists of monomers and linear polymers made by the head-to-tail polymerization. The law of mass action is assumed, where the binding constant a of monomer to i -mer is independent of the degree of polymerization i . Then, molar concentration λ_i of i -mer is related to monomer concentration λ by the equation: $\lambda_i = (a\lambda)^i / a$. Here, λ is determined by the

relation

$$N = \sum_i N_i = \lambda / (1 - a\lambda)^2, \quad 5$$

where N is the total concentration of monomer unit. The specific Kerr coefficient of a solution of monomers which have the anisotropy of induced polarizability α_i , and the permanent dipole moment μ_i is given by the equation,

$$B/C = A \left\{ \frac{\alpha_i}{KT} + \left(\frac{\mu_i}{KT} \right)^2 \right\}, \quad 6$$

where A is a constant determined by the molecular parameters (10). If the axial ratio of the monomer is larger than 7, A and α_i are numerically independent of the axial ratio. The anisotropy of induced polarizability α_i and the permanent dipole moment μ_i of i -mer are considered to be proportional to i . Therefore, if it is assumed that each particle in solution is independently oriented by the applied electric field, the specific Kerr coefficient of the solution is written as follows,

$$B/C = \left(\alpha \frac{\sum_i i N_i}{N} + \beta \frac{\sum_i i^2 N_i}{N} \right) = \left\{ \alpha \frac{1 + a\lambda}{1 - a\lambda} + \beta \frac{1 + 4a\lambda + a^2\lambda^2}{(1 - a\lambda)^2} \right\}, \quad 7$$

where α and β are constants determined by electrical and other molecular parameters of a monomer. The ratio Φ of the contribution of permanent dipole to that of induced dipole

is expressed as $\frac{\beta \sum_i i^2 N_i}{\alpha \sum_i i N_i}$ which is rapidly in-

creased with aN . Fig. 4 shows $\frac{1 + a\lambda}{1 - a\lambda}$ and

$\frac{1 + 4a\lambda + a^2\lambda^2}{(1 - a\lambda)^2}$ as functions of aN . Although

tropomyosin certainly has a permanent dipole moment, the measured specific Kerr coefficient changes with the protein concentration in a way similar to the calculated curve for particles having only induced dipole. If both the head-to-tail association and the head-to-head or the antiparallel side-by-side association take place at random, the average permanent dipole moment of i -mer is not given by $i\mu_i$, but given by $\sqrt{i} \mu_i$. In this case, if the axial ratios of the particles are sufficiently large, the specific Kerr coefficient

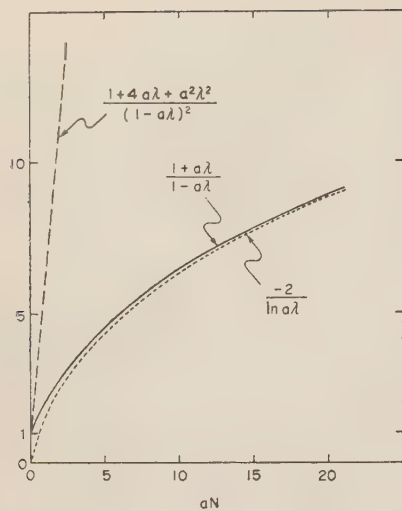


FIG. 4. The curves of $\frac{1+a\lambda}{1-a\lambda}$, $\frac{1+4a\lambda+a^2\lambda^2}{(1-a\lambda)^2}$ and $\frac{-2}{\ln a\lambda}$ as functions of aN .

of the solution is expressed,

$$\frac{B}{C} = \left(\alpha \frac{1+a\lambda}{1-a\lambda} + \beta \frac{1+a\lambda}{1-a\lambda} \right). \quad 8$$

The electric birefringence due to permanent dipole has the same relation to aN as that due to induced dipole, and Φ becomes independent of aN . This is in good agreement with the experimental facts. On the other hand, it is also evident that the aggregated tropomyosins have very large axial ratios. Hence, it is concluded that the polymerization of tropomyosin molecules is not only due to the head-to-tail association, but also due to the head-to-head or antiparallel side-by-side association, by which permanent dipole moment is canceled in part. Recently, from experiments of dielectric dispersion, Wada (15) presented a similar model of association in poly- γ -benzyl-L-glutamate solution.

The average length calculated from the decay time of birefringence in our experiment may be regarded as the length of the associated tropomyosins which make the maximum contribution in solution. On the other hand, as described previously, the contribution to birefringence by i-mers associated at random is proportional to $i^2(a\lambda)^i$. Then, the degree of polymerization $i_{\max}$ of the polymers which

make the maximum contribution to birefringence is given by $\frac{-2}{\ln a\lambda}$. The dotted line in

Fig. 4 shows $\frac{-2}{\ln a\lambda}$ as a function of aN . This curve is in qualitative agreement with the observed curves of the average length of tropomyosin particle L shown in Fig. 3. If it is assumed that the association is of a linear type and the monomer length is $400\text{\AA}$, the degree of association can be given as $L/400$. Then, the following equation is obtained,

$$\frac{L}{400} = \frac{-2}{\ln a\lambda}. \quad 9$$

By the use of this equation and the equation 5, binding constant a can be obtained. As shown in Fig. 5A, the value of a in the salt-free condition is very large and it gradually decreases with increasing salt concentration. This decrease of binding constant by salt is naturally expected if the polymerization is due to the electrostatic interaction. The

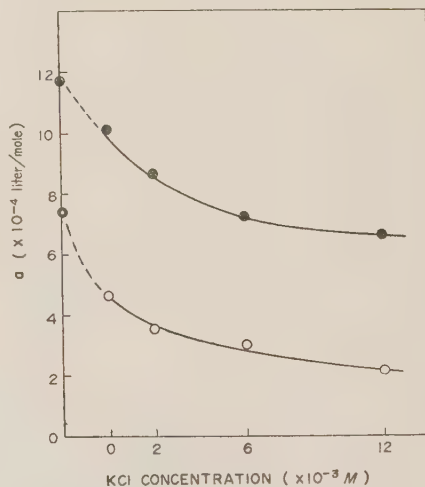


FIG. 5A. Plots of the binding constant (a) against KCl concentration in the presence of 2 mM phosphate buffer.

● and ○: at the protein concentration 2.4×10^{-4} g./ml. ($=4.55 \times 10^{-6} M$ in monomer unit), ○ and ⊙: at the protein concentration 1.9×10^{-3} g./ml. (⊙ and ⊙: in the absence of buffer). The binding constant was calculated from the average particle length by the use of the equation 9.

addition of urea also greatly decreases the binding constant as shown in Fig. 5B. As seen in these figures, the binding constant

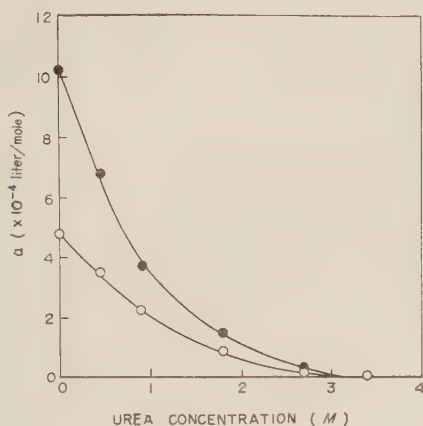


Fig. 5B. Plots of the binding constant (a) against the urea concentration. ●: at the protein concentration 2.4×10^{-4} g./ml., ○: at the protein concentration 1.9×10^{-3} g./ml.

estimated at low protein concentration is high in comparison with that at high protein concentration. This fact means that the binding constant between tropomyosin monomer and tropomyosin polymer actually decreases with the degree of polymerization.

Depolymerization by Increasing Temperature—The Kerr effect was measured at various temperatures. The plots of the specific Kerr coefficient and the estimated particle length against temperature are shown in Fig. 6.

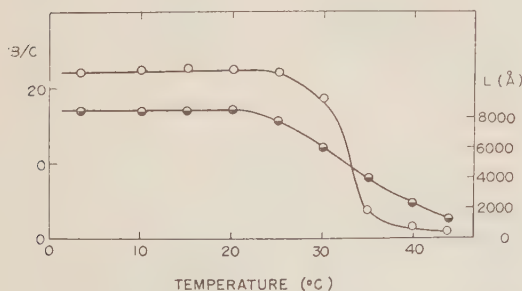


Fig. 6. Plots of the specific Kerr coefficient (B/C) and the particle length (L) against temperature. pH 8.9. Protein concentration 3.9×10^{-3} g./ml. ○: B/C , ●: L .

These quantities decrease abruptly above 30°C . This dissociation of tropomyosin molecules is completely reversible. Logarithm of the estimated binding constant does not have a linear relation to the inverse of temperature. It is likely that dissociation by increasing temperature is due to a transition in the structure of tropomyosin molecule.

pH Change—We examined the dependence of Kerr effect on pH to obtain some information of charges on tropomyosin molecule. Fig. 7 shows the specific Kerr coefficient at a fixed protein concentration as a function

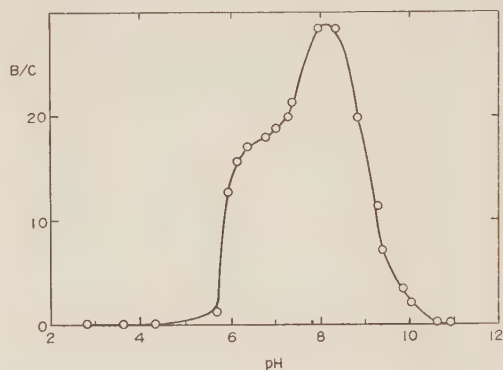


Fig. 7. Plot of the specific Kerr coefficient (B/C) against pH. Protein concentration 3.9×10^{-3} g./ml. Temperature 13°C . The pH was adjusted by the addition of small amount of KOH or HCl.

of pH. The estimated particle length is also given by a similar curve. When pH is increased from the isoelectric point to 8.5, the birefringence increases gradually and above pH 9.0 the birefringence begins to decrease. At pH higher than 10.5 and at acid pH, the specific Kerr coefficient takes smaller values than that obtained previously by infinite dilution at neutral pH.

DISCUSSION

Through the measurement of Kerr effect, it is concluded that aggregated tropomyosin molecules are depolymerized to monomers by infinite dilution of solution even under the salt-free condition. The remarkable depolymerization is observed only at very low concentration of tropomyosin. Therefore, the

measurements (2, 9) of light scattering and osmotic pressure which were made only at concentration higher than 4×10^{-4} g./ml., could not find this depolymerization phenomenon. Recent measurements of light scattering and viscosity by Ooi *et al.*,* however, have given new data which support our results. The magnitude of the permanent dipole moment can be determined from the specific Kerr coefficient through the equation 6, where the value of constant A is calculated from the following parameters; the refractive index of solvent, the refractive index of tropomyosin, the specific volume ($=0.71$) of tropomyosin, and Φ ($=2$). Since the specific Kerr coefficient at infinite dilution of solution is about $0.3 \text{ cm}^4/\text{g. volt}^2$, the permanent dipole moment of tropomyosin monomer is calculated to be about 390 Debye unit. The average value of permanent dipole increases with the concentration. For example, in the solution of protein concentration 4×10^{-3} g./ml. and phosphate buffer 2 mM, it becomes about 2,800 Debye unit. The presence of permanent dipole has been verified also in such biological macromolecules as fibrinogen (16), poly- γ -benzyl-L-glutamate (17), and the Holmes Rib Grass strain of tobacco mosaic virus (18) by the use of electric birefringence technique. It is not decided whether the observed permanent dipole of tropomyosin is mainly due to the asymmetric distribution of ionized amino acid residues or due to the polarity upon the CO...HN hydrogen bond.

In the analysis of the relation between electric birefringence and concentration of tropomyosin, the theory of Kerr effect established only in dilute solution, has been applied to the finitely concentrated tropomyosin solutions. In fact, the steric hindrance and the electrostatic interaction among tropomyosin particles must exert an influence upon orientation of the particles by applied electric field. Therefore, the previously described analysis by which we can obtain the conclusion upon the type of association may be a tentative one. In order to know the general feature

of association, this analytical method seems to be available, and will be applicable to other asymmetric macromolecular systems showing electric birefringence.

It is found that at pH lower than 6.0 and pH higher than 9.0 the birefringence of tropomyosin decreases remarkably on account of depolymerization. On the other hand, it is ascertained from measurement of optical rotatory dispersion that, in the pH range from 2.0 to 10.5, the helical content of tropomyosin molecule is kept constant (about 85 percent in our case) and only at pH higher than 11.0, it begins to decrease. Therefore, the depolymerization within the pH range 9.0–10.5 takes place without change of the helical structure of each tropomyosin molecule. In this pH range, such residual basic groups as lysine and tyrosine change their charges. The decrease of birefringence at pH about 6.0 is also considered to be due to the change in charges of acidic and histidic groups of tropomyosin. Consequently, it is likely that the charge pattern of tropomyosin molecule plays an important role in the polymerization mechanism. As pointed out by Bailey (8), the charged groups themselves may take part in the binding among tropomyosin molecules. As another possibility, the change of the charge distribution may indirectly give an influence on the ability of binding through the change of the tertiary structure of each tropomyosin molecule. The abrupt depolymerization above 30°C is not associated with the change of the helical content of tropomyosin and, therefore, it may also be attributable to the partial destruction of the tertiary structure by increasing temperature.

SUMMARY

1. For the purpose of investigating the polymerization mechanism, the electric birefringence of rabbit tropomyosin was measured at various conditions of solutions by the use of the rectangular and reversing pulse method. Electric dipole moments and lengths of monomers and polymers of tropomyosin were determined by the analysis of transient elect-

* Ooi, T., Mihashi, K., and Kobayashi, H., unpublished.

ric birefringence behaviors.

2. It was found that the aggregated tropomyosin molecules were completely depolymerized to monomers by infinite dilution even under salt-free condition and that tropomyosin monomer had a permanent dipole moment of about 390 Debye unit at neutral pH.

3. Theoretical analysis was successfully made on the relation between the birefringence and the tropomyosin concentration under the assumption of the reversible linear polymerization. As a result, it was concluded that the polymerization was not only due to the head-to-tail association but also due to the head-to-head or antiparallel side-by-side association, by which the permanent dipole of polymer was canceled in part. Also, it was shown that the binding constant between two tropomyosin molecules was decreased with increasing concentration of salt or urea.

4. At temperature higher than 30°C, associated tropomyosins were abruptly depolymerized and birefringence also decreased. At pH lower than 6.0 and at pH higher than 9.0, birefringence remarkably decreased.

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Metabolism of Organic Acids in *Rhodopseudomonas palustris* in Light and Dark

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Utilization by purple bacteria of carboxylic acids as H-donors in respiration and photosynthesis has been the subject of repeated investigations. The metabolic paths of these acids have also been studied by some workers (1). Eisenberg, investigating the oxidation of the members of TCA cycle in *Rhodospirillum rubrum*, concluded that the cycle constitutes the main metabolic path in the purple bacterium (2). Ormerod investigated the consumption of various H-donors such as succinic, fumaric and malic acids, accompanying photosynthetic carbon dioxide fixation in *Rhodospirillum rubrum* (3).

In the previous work, the present author investigated the metabolism of lactic and pyruvic acids in *Rhodopseudomonas palustris*, with special reference to the effect of light in modifying the oxidative and assimilatory processes involved (4).

The purpose of the present work was to extend the study, using the same bacterium, to other related organic acids (α -ketoglutaric, succinic, fumaric and malic), by following, in the dark and light, their assimilation into cell material and decomposition to carbon dioxide. For succinic and fumaric acids, in particular, studies were made of the fates of different carbon atoms in molecules by using C^{14} -labeled substrates. Based on the results obtained in the present and earlier studies, a general scheme will be presented for the mode of metabolism of these organic acids occurring in the bacterium.

MATERIALS AND METHODS

Bacterial Cells—A strain of *Rhodopseudomonas palustris* was grown for 48 hours at 27°C in a peptone-

lactate medium under constant illumination (about 3,000 lux) (4). The harvested cells were suspended in a phosphate buffer ($M/20$, pH 7.0) and used for experiments.

Labeled Compounds—Uniformly labeled fumaric acid was prepared from labeled glucose through fermentation according to the procedure devised by Takahashi*. The labeled glucose was derived from $BaC^{14}O_3$ through photosynthesis by green leaves of *Aster tataricus*, and then subjected to fermentation by *Rhizopus nigricans* to obtain fumaric acid.

1,2,3,4- C^{14} -succinic acid was obtained by reduction of labeled fumaric acid with zinc and phosphoric acid. The resulting product was recrystallized from water solution.

1,4- C^{14} -succinic acid (carboxyl-labeled) was prepared through fixation of $C^{14}O_2$ by *Bacterium succinicum* according to the method of Takahashi (5).

Procedures—The reactions were carried out in a Warburg vessel modified for the purpose as described in the previous paper (4). The composition of the reaction medium, unless otherwise stated, was as follows: bacterial suspension, 1.0 ml.; phosphate buffer, 1.0 ml. ($M/20$, pH 7.0); substrate, 0.5 ml. ($10 \mu\text{mole/ml.}$); and water to make a total volume of 5 ml. In most of the experiments 0.5 ml. of 5% potassium hydroxide solution was placed in the center well of the vessel. Reaction temperature was 27°C and the gas space in the reaction vessel was air. A bank of incandescent lamps (about 4,000 lux) was used as light source.

The reaction was started by adding the substrate to the reaction mixture, and stopped at the desired time by injection of 0.5 ml. of 35% trichloroacetic acid (TCA) into the vessel by means of a syringe through a rubber stopper in the side opening of the vessel.

The carbon dioxide evolved during the reaction was fixed with potassium hydroxide solution in the

* Personal communication

center well, and precipitated as barium carbonate. The determination for radiocarbon in carbonate and in cell material was carried out using a thin-windowed Geiger-Müller tube (0.9 mg./cm.²), corrections being made for self-absorption of the sample.

The oxygen-uptake was measured with a Warburg manometer under the same experimental conditions as described above.

RESULTS

Oxygen-uptake in the Presence of Different Substrates as Influenced by Light—The rate of oxygen-uptake by the resting cells was found to be accelerated by the addition of succinic and malic acids, both in the light and in the dark (Fig. 1). A marked suppression in rate

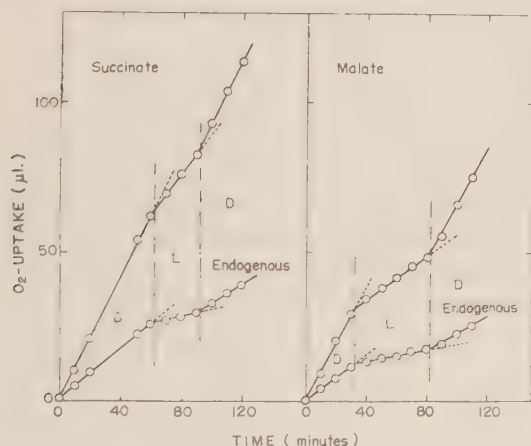


FIG. 1. Oxygen-uptake in light and dark with and without succinate or malate as substrate. For compositions of reaction media see text (100 μ moles of substrate per each vessel).

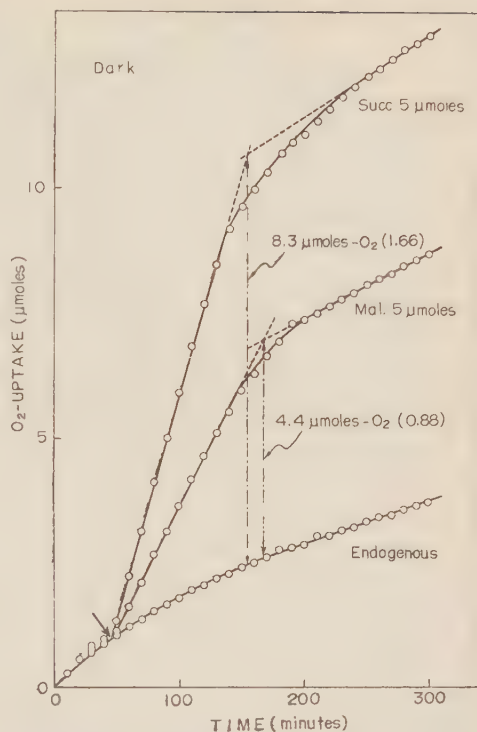


FIG. 2. Oxygen-uptake in dark in the presence and absence of succinate or malate as substrate. For composition of reaction medium see text. The arrow in the figure indicates the time of addition of substrates.

of oxygen-uptake was always observed on illumination in the presence, as well as in the absence, of the added substrates, a finding which is in accord with the results of previous investigators (1, 4, 6). Experiments were also carried out to determine the total amounts of oxygen consumed for the exhaustion of added substrates, the time of exhaustion being recognized by an abrupt decrease of the rate of oxygen-uptake to attain the original endogenous level (Fig. 2). In Table I are summarized the molar ratios thus determined of surplus oxygen absorbed to substrate consumed. On the assumption of "complete" oxida-

tion (to the level of carbon dioxide and water), the ratios in question are calculated to be 4.0, 3.5, 3.0, 3.0 and 2.5 for α -ketoglutaric, succinic, malic, lactic and pyruvic acids, respectively. The values actually found were: in the dark 1.5, 1.5, 0.9, 1.2 and 0.6, and in the light 1.1, 0.7, 0.5, 0.4 and 0.0, respectively, indicating that, both in light and dark, all the substrates tested were oxidized quite "incompletely".

Experiments with 1,2,3,4-C¹⁴-labeled Succinic Acid—Uniformly C¹⁴-labeled succinic acid was given to the bacterial cells in order to investigate the fate of carbon atoms in the acid molecule. After stopping the reaction by the addition of TCA-solution, the measurement of radioactivity was made on (i) the carbon dioxide evolved and fixed in the potassium hydroxide solution in the center well, (ii) the bacterial cells collected (and washed) by cen-

TABLE I

Molar Ratios of Oxygen Used to Substrate Consumed in the Light and Dark

The data for lactic and pyruvic acids are those obtained in previous experiments (4).

substrate	condition of incubation	mole O ₂ -consumed/mole substrate consumed		
		calculated ¹⁾	observed	
				mean
α -ketoglutarate	dark	2.0	1.14—2.14	1.51
	light	1.5	1.08—1.18	1.13
succinate	dark	1.5	1.02—1.72	1.45
	light	1.0	0.55—0.89	0.73
malate	dark	1.0	0.70—1.14	0.92
	light	0.5	0.32—0.56	0.45
lactate	dark	1.0		1.15
	light	0.5		0.37
pyruvate	dark	0.5		0.59
	light	0.0		0.03

1) For the method of computation see text.

trifugation of the reaction mixture, and (iii) the supernatant solution, combined with the washings of the bacterial cells. Judging from the results of paper-chromatographic tests, major part of radioactivity detected in (iii) resulted from the residual succinic acid remaining in the reaction mixture, other TCA-soluble substances extracted from the cells by the acid treatment being insignificant in quantity (actually, less than one-tenth of the total activity incorporated in the cells). In the consideration of the distribution of carbon atoms, therefore, the TCA-soluble fraction of the reaction products was neglected.

A typical result with 1,2,3,4- C^{14} -labeled succinic acid as substrate is presented in Fig. 3. From a single sight of the figure, the surprisingly high efficiency of incorporation of substrate carbon in cell material will be evident. In the dark, an amount one-half that evolved as carbon dioxide was incorporated. The light increases the efficiency to a still higher level, giving a ratio of incorporation and discharge as high as 2:1. As measured by the sum of radioactivities in carbon dioxide and cell material, the total rate of substrate consumption, was not signi-

ficantly altered by illumination. From the experimental results it was computed that out of four atoms in a succinic acid molecule,

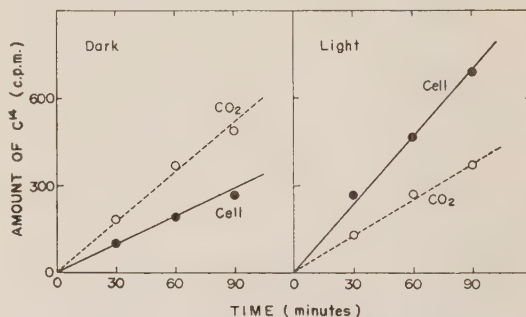


FIG. 3. Evolution of $C^{14}O_2$ and incorporation of C^{14} into cell material from 1,2,3,4- C^{14} labeled succinate in the light and dark. For composition of reaction medium see text. Specific activity of substrate: 248 c.p.m. per μ mole succinate (5 μ moles of substrate per each vessel).

1.6 were incorporated in cell material and 2.4 were discharged as carbon dioxide in the dark, while 2.5 were incorporated in cell material and 1.5 were given off as carbon dioxide in the light (Table II).

Experiments with 1,4- C^{14} -labeled Succinic Acid—An experiment similar to the one described

TABLE II

Evolution of $C^{14}O_2$ and Incorporation of C^{14} into Cell Material from C^{14} -labeled Succinate and Fumarate in the Light and Dark

substrate	condition of incubation	C^{14} in cell material	C^{14} in CO_2 evolved
1, 2, 3, 4- C^{14} succinate	dark	40% (1.6 atom/mole)	60% (2.4 atom/mole)
	light	64% (2.5 atom/mole)	36% (1.5 atom/mole)
1, 2, 3, 4- C^{14} fumarate	dark	34% (1.4 atom/mole)	66% (2.6 atom/mole)
	light	57% (2.3 atom/mole)	43% (1.7 atom/mole)
1, 4- C^{14} succinate	dark	5% (0.1 atom/mole)	95% (1.9 atom/mole)
	light	25% (0.5 atom/mole)	75% (1.5 atom/mole)

Each figure in the table represents the mean of two to three experiments. For method of computation see text.

above was performed with 1,4- C^{14} -labeled succinic acid. The results are presented in Fig. 4 and Table II. It will be seen that most of the carboxyl carbon of succinate was converted into carbon dioxide, which was especially the case in the dark experiments, where 90 per cent of 1 and 4 carbon was recovered in the evolved carbon dioxide. Here also the light caused a marked increase in the yield of cellular incorporation, without significantly influencing the total rate of substrate consumption. The balance of cell- and CO_2 -carbon is thus shifted, in the light, in favor of the former; 1:3, in the light, compared with 1:10 in the dark. Although no particular experiment has been carried out in the present study to follow directly the fate of the methylene carbon, it is concluded from the comparison of these sets of experimental results that, under illumination, all the methylene carbons of succinic acid pass into the cell material, while in the dark one-fourth of the same carbon is converted into carbon dioxide.

1,2,3,4- C^{14} -labeled Fumaric Acid—The experiment using uniformly labeled fumaric acid as substrate, gave the results presented in Fig. 5. Here also, a high efficiency of incorporation, as manifest in the ratio of cell- C^{14}/CO_2 - C^{14} , was observed, especially under illumination (Fig. 5).

Effect of (non-labeled) Carbon Dioxide—

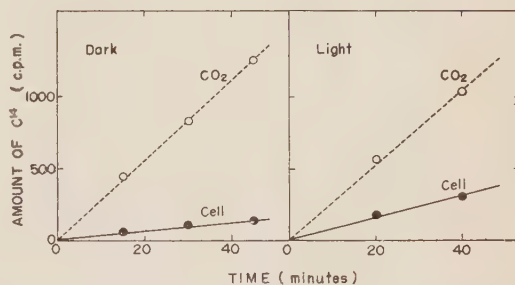


FIG. 4. Evolution of $C^{14}O_2$ and incorporation of C^{14} into cell material from 1,4- C^{14} -labeled succinate in the light and dark. For composition of reaction medium see text. Specific activity of substrate: 300 c.p.m. per μ mole succinate (5 μ moles of substrate per each vessel).

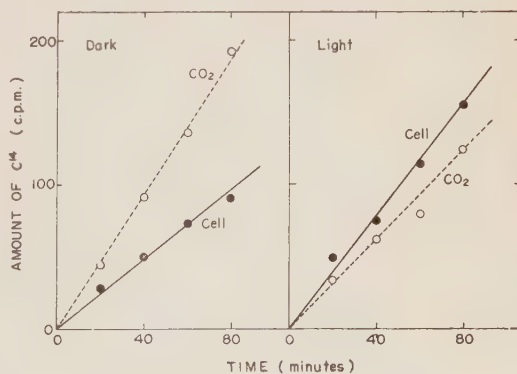


FIG. 5. Evolution of $C^{14}O_2$ and incorporation of C^{14} into cell material from 1,2,3,4- C^{14} -labeled fumarate in the light and dark. For composition of reaction medium see text. Specific activity of substrate: 124 c.p.m. per μ mole fumarate (5 μ moles of substrate per each vessel).

TABLE III

Effect of Non-labeled Carbon Dioxide on Incorporation of C¹⁴ from 1,4-C¹⁴-Succinate into Cell Material

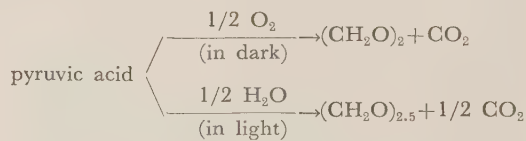
	light			dark
	(a) with 5% CO ₂ in gas space	(b) without CO ₂ and with KOH in center well	(c) without CO ₂ and without KOH in center well	with air and with KOH in center well
C ¹⁴ in cell material	25%	24%	34%	10%
C ¹⁴ in CO ₂	75%	76%	66%	90%

Another set of experiments was carried out to investigate whether the above-described incorporation of radiocarbon in the light was not due to a secondary (photosynthetic) fixation of carbon dioxide produced from the labeled substrate. For this purpose, three parallel experiments were performed in the light using 1,4-C¹⁴-labeled succinate as substrate; (a) with provision of (non-labeled) carbon dioxide to the reaction medium, (b) without carbon dioxide, but with potassium hydroxide in the center well, and (c) the same, but without potassium hydroxide. As a control a dark experiment was also performed with air in the gas space and with potassium hydroxide in the center well. The reaction mixtures were incubated at 27°C and the radioactivity in cell material and in carbon dioxide evolved was measured at intervals. The results are presented in Table III, in which each figure represents a mean of the values obtained in two or three experiments. As may be seen, the distribution of C¹⁴ in the cell material and carbon dioxide evolved was the same in the runs of (a) and (b), while more C¹⁴ went into cell material in (c). The fact that the C¹⁴-distribution was the same in (a) and (b) shows that when potassium hydroxide was present in the center well—a condition which was usually the case in experiment—there occurred no significant reassimilation of the carbon dioxide evolved by the breakdown of substrate. The higher value for the cellular radiocarbon observed in (c) may be interpreted as indicating that

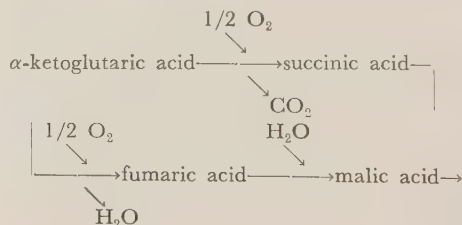
in the absence of the carbon dioxide absorber (potassium hydroxide) some portion of the carbon dioxide evolved by the breakdown of succinate was captured by photosynthesis.

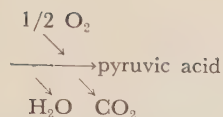
DISCUSSION

It is notable that all the organic substances investigated in the present study are utilized with high efficiencies, and especially in the light, in the aerobic metabolism of *Rhodospseudomonas palustris*. In a previous work (4) it has been shown that the bacterium can assimilate, with an equally high efficiency, pyruvate under light-anaerobic and dark-aerobic conditions. While the assimilation in the light could proceed under the anaerobic condition (*i.e.*, in the complete absence of respiratory metabolism), the assimilation in the dark was only possible under the aerobic condition. The material balance in this case was found to be as follows:

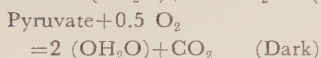
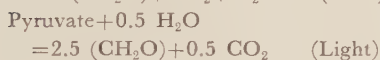
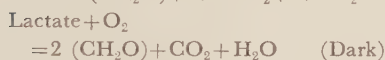
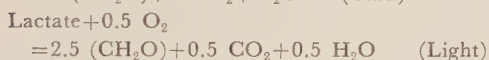
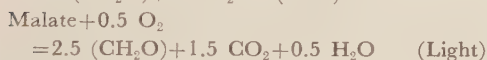
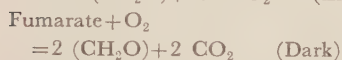
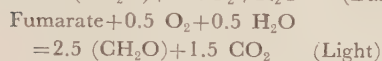
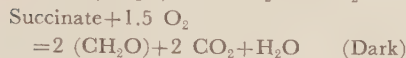
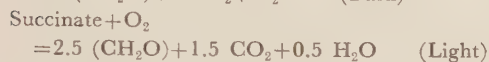
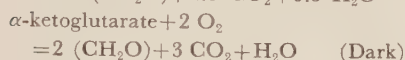
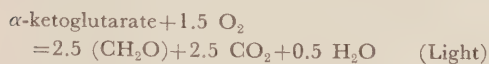


If we combine the above reactions with the well-known sequence of the reactions:





we may write the following formulae for the metabolism of the various organic acids occurring in the light and in the dark:



The agreement between the values given in these formulae and those obtained experimentally (Table I) may be regarded as satisfactory, when we make allowance for the fluctuations of values to be encountered in this kind of experiment. A rather significant deviation from the theory was found in the cases of α -ketoglutarate (both in light and dark) and of succinate (in light). The formulae given for succinate and fumarate can also be checked with the data given in Table II. So far as the results of the light experiments are concerned, the distribution of C^{14} obtained with uniformly-labeled (1,2,3,4- C^{14}) succinate and fumarate was in good agreement with the relations expressed by the formulae. The deviation from the theory in the dark experiments (Table II) may be due to the occurrence of some side reactions such

as the respiratory oxidation of the substrates. The results of experiments using 1,4- C^{14} -labeled succinate as substrate (Table II) give evidence that in the dark practically all the carbon dioxide evolved from succinate was derived from the carboxyl carbons, and that in the light, some portion of the carboxyl carbon was incorporated in cell material. Most probably, the same may be true in the case of fumarate.

SUMMARY

1. Using *Rhodospseudomonas palustris* as material, investigations were made of the aerobic metabolism, in light and darkness, of α -ketoglutaric, succinic, fumaric and malic acids.

2. The molar amounts of oxygen taken up for one mole of each substrate consumed were found to be as follows:

In light: α -ketoglutaric, 1.13; succinic, 0.73; malic, 0.45.

In dark: α -ketoglutaric, 1.51; succinic, 1.45; malic, 0.92.

3. Experiments with uniformly- C^{14} -labeled fumarate and succinate showed that the distribution of their four carbons in cell material and in carbon dioxide evolved was modified by light—and—dark conditions as follows:

In light: 1.5 atoms per mole in CO_2 , 2.5 atoms per mole in cell material.

In dark: 2.5 atoms per mole in CO_2 , 1.5 atoms per mole in cell material.

4. Using 1,4- C^{14} -labeled succinate as substrate, it was revealed that the two carboxyl- C^{14} were distributed to cell material and carbon dioxide evolved in the following ratio:

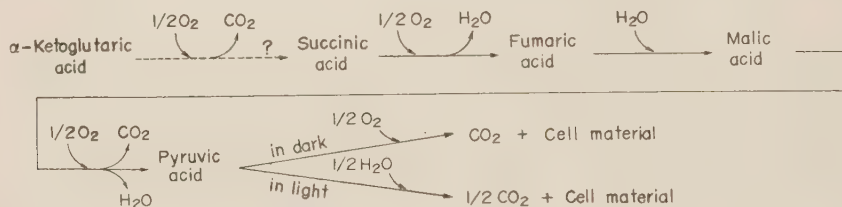
In light: 1.5 atoms per mole in CO_2 , 0.5 atoms per mole in cell material.

In dark: 1.9 atoms per mole in CO_2 , 0.1 atom per mole in cell material.

5. In the light, and when potassium hydroxide was applied as carbon dioxide absorber, the efficiency of incorporation of substrate (succinate) carbon into cell material was the same as that in the presence of sufficient amounts of non-labeled carbon dioxide in the reaction mixture, indicating

that in the presence of the carbon dioxide absorber, the respiratory $C^{14}O_2$ produced from the substrate is not assimilated photosynthetically.

6. Based on the present data and those obtained earlier with pyruvate, the following sequence of reactions was assumed for the metabolism of the organic acids in *Rhodospseudomonas palustris*.



It is a pleasure for the author to express his thanks to Prof. H. Tamiya for his continuous guidance and keen interest throughout this work. His thanks are also due to Prof. A. Takamiya for helpful advice and kind suggestions. The author is also indebted

to Dr. H. Takahashi and Dr. S. Katoh for their help to prepare the radioactive compounds.

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4-O-Acetyl Mycarose

A New O-Acetyl Sugar Obtained from Leucomycin Minor Components

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A great variety of sugars esterified with sulfate or phosphate, is known as cell constituent and metabolite. However, there have been no report on the presence of sugars, whose hydroxyl group is esterified with carboxylic acid, except 6-O-acetyl glucose as a metabolite of *Bacillus megaterium* (1, 2) and 4-O-isovaleroyl mycarose as a constituent of two macrolide antibiotics, magnamycin (3) and leucomycin A₁ (4)*.

In this paper, is described the presence of another O-acyl sugar, 4-O-acetyl mycarose, as a sugar component of leucomycin B, which was a mixture of minor components in the antibiotic complex produced by *Streptomyces kitasatoensis* Hata (5-7).

EXPERIMENTALS

Materials—Leucomycin complex used as starting material, was a commercial bulk, Lot. No. 9 LM-44, which was kindly offered from the Research Institute, Toyo Jozo Co., Ltd.

The complex was previously resolved into A group-rich and B group-rich fractions, by the fractional precipitation at different pH and the fractional extraction with benzene (see Scheme I (9)). From each fraction, leucomycin components A₁ and A₂ or B₁, B₂, B₃ and B₄ were isolated by further minute resolution with the use of counter current distribution technique (10). The typical distribution diagram and the properties of each component are represented in Fig. 1 and Table I respectively.

Isolation of O-Acyl Sugar from Acid-Hydrolysate of Leucomycin—Leucomycin component (100 mg.) was dis-

* As a glycoside, the presence of another sugar ester, 1-O- β -benzoyl-D-glucose, was isolated from the solid excretion on the necks of *Blattidae* (8).

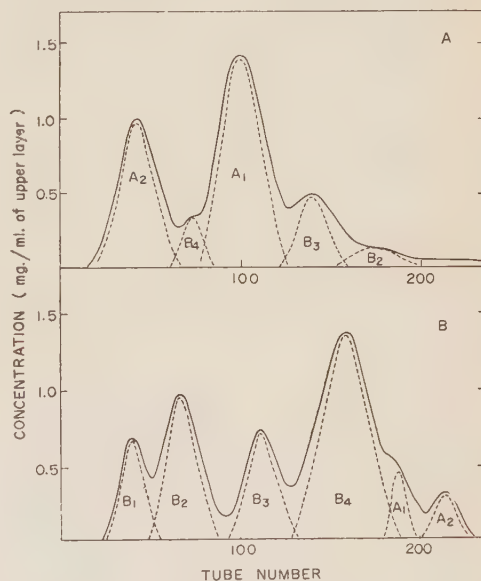


FIG. 1-(A). Counter current distribution of A group-rich fraction using a solvent system composed of benzene, chloroform, methanol and water (420 transfer).

FIG. 1-(B). Counter current distribution of B group-rich fraction using a solvent system composed of chloroform, acetone and water (250 transfer).

solved into *N*/2 hydrochloric acid (1 ml.) and the solution was allowed to stand for 24 hours at room temperature. Thereafter, the solution was neutralized to pH 4 and was extracted with ether (three times, 10 ml. each). The ether extracts contained a sugar derivative which was colored with vanilline-perchloric acid reagent (11) and possessed an intense infrared absorption at 1730 cm⁻¹.

Alkaline Hydrolysis of O-Acyl Sugar—Alkaline hydrolysis of the above-mentioned sugar derivative (40

TABLE I
Properties of Leucomycin A₁, A₂, B₁, B₂, B₃ and B₄

	A ₁	A ₂	B ₁	B ₂	B ₃	B ₄
Molecular Formula	C ₄₆ H ₈₁ NO ₁₇	C ₆₅ H ₁₁₁ NO ₂₂	C ₃₅ H ₅₉ NO ₁₃	C ₃₈ H ₆₅ NO ₁₆	C ₃₄ H ₅₃ NO ₁₃	C ₃₈ H ₅₉ NO ₁₆
m.p.	135.0–138.0°	142.0–144.0°	214.5–216.5°	214.0–216.0°	216.0–217.0°	221.0–223.8°
$\lambda_{\text{max}}^{N/1000 \text{ HCl}}$	233	231	234	234	234	233
(ϵ_{max} ; $\times 10^4$)	(2.45)	(1.98)	(2.24)	(2.47)	(2.32)	(2.45)
Antibacterial activity (unit/mg.)	1000	910	100	215	570	700
pK'a	7.1	7.1	6.6	6.7	6.7	6.8

mg.) was performed in *N* sodium hydroxide (1 ml.) at room temperature for 24 hours. The hydrolysate, after being neutralized, was concentrated to dryness *in vacuo*, and the residue was extracted with ethanol. The extract contained a sugar which was also positive to vanilline-perchloric acid reagent but possessed an infrared absorption spectrum quite different from the original sugar derivative (no absorption at 1730 cm⁻¹).

Hydroxamic Acid from O-Acyl Sugar—Another portion of the above-mentioned sugar derivative (40 mg.) was dissolved into methanolic solution of hydroxylamine (salt-free, 2 ml. containing 0.28 g. NH₂OH) and the solution was heated at 90°C for 30 minutes. The filtrate from the acidified solution was directly used as a sample of paper chromatography (12).

Periodate-Oxidation of O-Acyl Sugar—The oxidation was achieved in 0.5% acetic acid at 20°C, with the uses of 0.25 mmole of sample and of 3.1 mM of sodium metaperiodate. After a given period, an aliquot of the solution was added with excess amounts of potassium iodide, and the liberated iodine was titrated with thiosulfate, to determine the remaining amount of the oxidant.

Paper Chromatographic Analysis—Sugars and the derivatives were analyzed by paper chromatography, using a freshly prepared mixture of *n*-butanol, acetic acid and water (4:1:5 v/v). The upper layer was the mobile phase, while the lower one was used for the previous equilibration of filter paper.

Spots of sugar and the derivatives were located by spraying vanilline-perchloric acid reagent and by heating at 95°C, under which the purple coloration had been reported to be specific for 2-deoxyaldose (11).

Identification of hydroxamic acids were performed by comparing their chromatographic *R_f* values developed with a mixture of *n*-butanol, acetic acid and water (4:1:1 v/v) and with *n*-butanol saturated with 3 *N* acetic acid (13), with those of authentic samples.

RESULTS AND DISCUSSION

Each leucomycin component (A₁, A₂, B₁, B₂, B₃ and B₄) produced reducing substance(s) on the treatment with *N*/2 hydrochloric acid at room temperature. The reducing product(s) migrated as a single spot on paper developed with a mixture of *n*-butanol, acetic acid and water, and colored with vanilline-perchloric acid reagent, which had been reported to be specific for 2-deoxyaldose (11). As intact leucomycin possessed no reducing group (14), these results seemed to indicate that all of the leucomycin components had acid-labile 2-deoxyaldoside linkage(s). As summarized in Table II, the 2-deoxyaldose derived from leucomycin B₂, B₃ and B₄ (*R_f* 0.86) differed from the 2-deoxyaldose obtained from leucomycin A₁, A₂ and B₁ (*R_f* 0.96) in their paper chromatographic behaviors. As to the color on paper with vanilline-perchloric acid reagent, there was also observed some difference between the both spots.* On the treatment with *N* sodium hydroxide at room temperature, however, the both 2-deoxyaldoses

* The color produced from O-acylated mycarose was too unstable to determine the absorption spectrum.

TABLE II
R_f of 2-Deoxyaldoses derived from Leucomycin
Components

2-Deoxyaldose from:	<i>R_f</i> ¹⁾	
	Before Alkaline	After Hydrolysis
Leucomycin Complex ²⁾	0.86, 0.96	0.78
Leucomycin A ₁	0.96	0.78
Leucomycin A ₂	0.95	0.78
Leucomycin B ₁	0.97	0.77
Leucomycin B ₂	0.86	0.78
Leucomycin B ₃	0.86	0.78
Leucomycin B ₄	0.87	0.78
4-O-Isovaleroyl-mycarose	0.97	
Mycarose	0.78	

1) Solvent system; *n*-butanol, acetic acid and water (4:1:5 v/v) Detection; vanilline-perchloric acid.

2) A mixture of A₁, A₂, B₁, B₂, B₃ and B₄.

likewise changed into another sugar, the color with the above-mentioned reagent and the

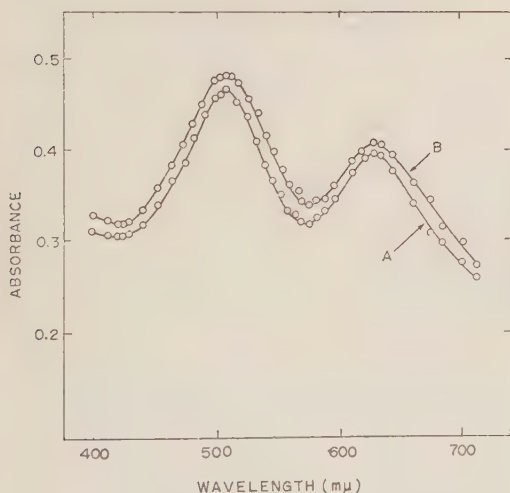


FIG. 2. Absorption spectrum of the color produced from mycarose with vanilline-perchloric acid reagent.

A: Mycarose obtained from leucomycin A₁ (4).

B: Mycarose obtained from leucomycin B₂.

These spectra were measured by direct spectrophotodensitometry of spot on paper chromatogram.

R_f of which could not be distinguished from those of mycarose (*R_f* 0.78: see Fig. 2). As the 2-deoxyaldose obtained from leucomycin A₁ (*R_f* 0.96) had been identified as 4-O-isovaleroylmycarose (4), another one derived from leucomycin B₂, B₃ and B₄ (*R_f* 0.86) seemed to be mycarose or a similar sugar esterified with an acid more hydrophylic than isovaleric acid.

The presumption was partially supported by analyzing their hydroxylaminolysates. On hydroxylaminolysis the both 2-deoxyaldoses gave substance(s) which gave characteristic color with ferric ion in acid solution, but their paper chromatographic analyses indicated the formation of acetyl hydroxamate from the 2-deoxyaldose of *R_f* 0.86 (from B₂, B₃ and B₄) and the formation of isovaleric hydroxamate from the 2-deoxyaldose of *R_f*

TABLE III

R_f Values of Acyl Hydroxamates Obtained from 2-Deoxyaldose Derivatives

O-Acyl 2-Deoxyaldose obtained from:	<i>R_f</i>	
	1	2
Leucomycin A ₁	0.96 ¹⁾	0.92
Leucomycin A ₂	0.95	0.92
Leucomycin B ₁	0.97	0.92
Leucomycin B ₂	0.68	0.61
Leucomycin B ₃	0.68	0.60
Leucomycin B ₄	0.67	0.60
Authentic Hydroxamate		
Formic	0.40	0.51
Acetic	0.68	0.60
Propionic	0.81	0.80
<i>n</i> -Butyric	0.96	0.86
<i>i</i> -Isovaleric	0.97	0.92

Solvent system; 1) *n*-Butanol, acetic acid and water (4:1:1 v/v)

2) *n*-Butanol saturated with 3 *N* acetic acid

Detection; Ferric chloride

1) The presence of isovaleroyl group had been confirmed by isolating isovaleric acid (as *S*-benzyl thiouronium salt, m.p., 153°C) from the alkaline hydrolysate of O-acyl 2-deoxyaldose.

0.96 (from A₁, A₂ and B₁)*. The result is shown in Table III. The presumed O-acetyl mycarose was actually isolated from a mixture** of leucomycin B₂, B₃ and B₄, by the mild acid hydrolysis.

O-Acetylmicarose (or the isomer), b.p., 65°C/1.5 mm Hg. Yield, 80 mg. from 500 mg. of a mixture of leucomycin B₂, B₃ and B₄ (about 75%). C; 52.94% H; 7.84%, calcd. for CH₃COO-C₇H₁₃O₅. C; 53.30% H; 8.04%, found.

The infrared absorption spectrum is shown in Fig. 3, together with 4-O-isovaleroylmycarose

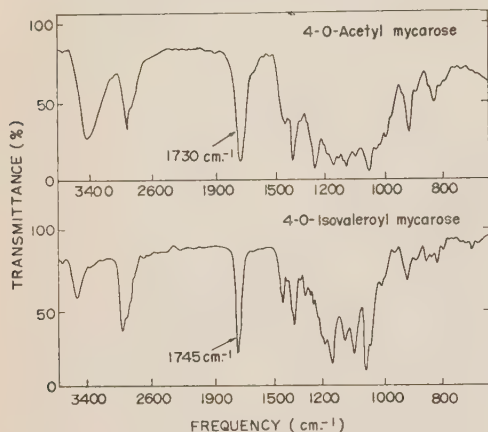


FIG. 3. Infrared absorption spectra of 4-O-acetyl mycarose and 4-O-isovaleroylmycarose.

(4). The presence of an intense infrared absorption at 1730 cm⁻¹ and the chemical composition suggested the presence of O-acetyl group in trideoxyheptose (C₇H₁₄O₄), instead of O-isovaleroyl group. On alkaline hydrolysis in preparative scale, the sugar acetate gave a free sugar, having no absorption at 1730 cm⁻¹.

The free sugar, m.p. 88°–89°C. Yield 50 mg. from 100 mg. of 4-O-acetylmicarose (75%), C; 46.41%, H; 9.39%, calcd. for C₇H₁₄O₄·H₂O. C; 46.38%, H; 9.18%, found.

The sugar could not be distinguished from mycarose either in the chromatographic behavior or the color with vanilline-perchloric

* The isomers and higher homologues of isovaleric hydroxamate also possessed similar *R_f* values.

** Due to the limited amounts of purified components, a mixture free from A and B₁ components (assayed by paper chromatography (15)).

acid reagent (Fig. 2), and the melting point did not depress on admixture with authentic mycarose, which had been isolated by the present author from leucomycin A₁ (4). As shown in Fig. 4, the infrared absorption spectrum was also just the same as that of mycarose.

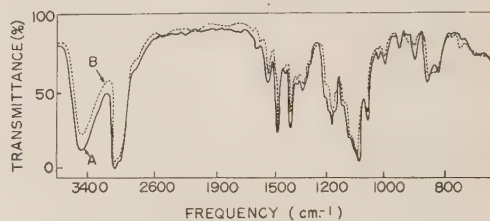


FIG. 4. Infrared absorption spectrum of mycarose.

A: Authentic sample (4).

B: Obtained from leucomycin B₂, B₃ and B₄.

Table IV summarizes the result on the periodate-oxidation of mycarose and the O-acyl derivatives. As being expected, mycarose

TABLE IV

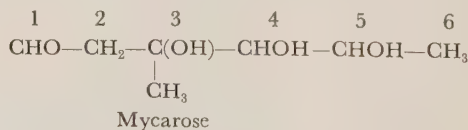
Periodate-Oxidation of Mycarose and the O-Acyl Derivatives

10⁻⁴ moles consumed per sugar mole

Mycarose	1.94
O-Acetyl-Mycarose	0.18
O-Isovaleroyl-Mycarose	0.08

In 5% acetic acid at 20°C, for 24 hours.

consumed 2 moles of the oxidant, while the O-acyl mycarose obtained from leucomycin B₂, B₃ and B₄, as well as 4-O-isovaleroylmycarose from leucomycin A₁, did not consume periodate under the conditions used. The fact indicates the presence of O-acetyl group at the C₄-position of mycarose.



As shown in Scheme I in previous paper

(9), leucomycin complex used was obtained from the broth of *Streptomyces Kitasatoensis* Hata by extracting with butylacetate at a slight alkaline pH. The step might permit an acyl migration between the O-isovaleroyl groups of leucomycin A₁, A₂ and B₁, and the O-acetyl group of solvent, giving leucomycin B₂, B₃ and B₄ as their artifacts. Such a possibility, however, had been already excluded by the direct isolations of B₂, B₃ and B₄ from the lyophilized broth with the use of elution chromatography from a column of Amberlite XE-64 (9).

The coexistence of O-acetyl (in B₂, B₃ and B₄) and O-isovaleroyl (in A₁, A₂ and B₁) mycarosides in metabolites of *Streptomyces* of the same strain, seems to be interesting. It is because that these leucomycin components have been presumed to be macrolide antibiotic similar to magnamycin whose mycarose moiety is acylated only with isovaleric acid (3). Recently, there appeared reports indicating that the aglycon part of macrolides seemed to be synthesized by a polymerisation of propionic acid or the methabolically related compound, such as succinyl or malonyl Co A (16). The relationship among the metabolism of acetic, propionic and isovaleric acids, might be an important factor, effecting the amount and composition of leucomycin complex. In this respect, it should be emphasized that a strain of *Streptomyces kitasatoensis* producing only O-isovaleroyl mycarosides (A₁ together with small amount of A and B) could be isolated from mutants of the original strain*.

* The strain is now used for the preparation of leucomycin ('Neoleucomycin') as A₁ has the highest antibacterial activity. (see Table I)

We are greatly indebted to Mr. T. Fujimoto, Mr. A. Take and Miss Y. Tsuyuki for their assistance in elemental analysis and functional group analysis.

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Studies on the Incorporation of P^{32} -Labelled Orthophosphate into the Phosphatides of Heart and Femoral Muscle of Rat

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After the first suggestion by Gemmil (1) that lipids might be important endogenous substrates in muscle in view of the respiratory quotient, considerable evidence has been obtained to indicate that fatty acids are utilized in many kinds of muscle (2~4). The result of further extensive studies by Neptune (5), in which the fall in total fatty acids in diaphragm could account for 100 per cent of the oxygen consumption but glycogen consumption was very small, indicated that lipids were the major source of endogenous substrates in the muscle. On the other hand, phosphatides are present more abundantly in the active muscles, such as heart or thoracic muscle of birds, than in ordinary skeletal muscle. This evidence suggests the necessity to evaluate the role played by muscle phosphatides. However, not only the metabolism but also the composition of muscle phosphatides has been scarcely studied. An attempt, therefore, has been made in this study to elucidate the content and composition of phosphatides and its turnover in heart and femoral muscle of rat.

In most cases, studies on the incorporation of P^{32} into phosphatides in various tissues, *e.g.* brain (6,8), avian salt gland (9), erythrocytes (10), and pancreas (7), were carried out by paper chromatography on silicic acid impregnated paper. However, by this method alone strict analysis is difficult as will be shown later, although the method is simple and less time-consuming.

The column chromatography on silicic acid and two kinds of paper chromatography were applied in this study, in the hope that comparative studies using various techniques

might cast some further light on problems in this field.

EXPERIMENTALS

Rats weighing 100–120 g. were killed by decapitation at various periods of time after an intraperitoneal injection of 600–1000 μ c of P^{32} -orthophosphate.

The excised heart and femoral muscles were put into an ice-cold 0.9% NaCl solution for about 2 minutes, then weighed after blotting the water and blood with a filter paper, and phosphatides were extracted as described in the following section.

Extraction of Phosphatides—The tissue (about 1 g.) was homogenized in a glass homogenizer with a 20 fold volume of chloroform-methanol 2 : 1, and centrifuged at $3000 \times g$ for 10 minutes. The residue was extracted once again with 10 ml. of the chloroform-methanol for 30 minutes at room temperature with occasional stirring, and the extracts were combined. To the combined extract of lipids 0.2 volume of 0.9 % NaCl was added, then the mixture was stirred and centrifuged. After removing the upper layer 0.3 volume of distilled water saturated with chloroform-methanol was added to the lower layer, stirred and centrifuged, then the water layer was removed. The solvent layer and white fluff at the interphase were taken together and dried under reduced pressure in an atmosphere of nitrogen at 37°C. The residue was extracted with 20 ml. of chloroform and filtered with a small filter paper and washed carefully with chloroform. The chloroform extract was again evaporated to dryness, and for paper chromatography on silicic acid impregnated paper it was dissolved in isoamyl-alcohol-benzene 1 : 1, for mild alkaline hydrolysis dissolved in carbon tetrachloride.

Column Chromatography with Silicic Acid—Silicic acid (Mallinckrodt, analytical reagent, 100 mesh, for chromatography) was thoroughly washed with methanol and fine powder was removed. After drying, silicic acid was activated at 110°C overnight. A glass tube with 1.0 cm. inside diameter by 15 cm. containing a

glass wool plug was packed with 5 g. of silicic acid suspended in 20 ml. of petroleum ether. The column was washed through successively with 20 ml. of petroleum ether, 20 ml. of chloroform-methanol 1:1, and 50 ml. of chloroform. A 10 ml. aliquot of the chloroform extract of lipids containing 0.8–1.2 mg. of P was passed through the column and eluted with the following solvents in the order given: chloroform, chloroform-methanol 15:1, 4:1, 2:1, 1:1, 4 and methanol. The flow rate was about 0.5 ml. per minute. Each fraction collected was 5 or 10 ml. and aliquots of each were taken for paper chromatography, for total P analysis and for radioactivity determination.

Paper Chromatography—Silicic acid impregnated paper was prepared by the method of Marinetti *et al.* (11). The isoamylalcohol-benzene solution of phosphatides, containing 20–70 μ g. of total P, was applied to the paper on the base line. The solvent system was diisobutylketone-acetic acid-water 40:25:5. The detection of spots and elution of phosphatides for quantitative analysis were carried out by the method of Marinetti *et al.* (12), and paper chromatographic analysis of mild alkaline hydrolysate by the method

of Dawson (13).

Phosphorus analysis was made by the method of Allen (14) or Horecker (15). All solvents used were of B grade (extra pure) and distilled once before use. Methanol was added (in 1%) to chloroform as preservative.

The identification of the components of phosphatides was made by the R_f values, rhodamine 6 G and ninhydrin staining and choline reactions.

The detection of ethanolamine in the acid hydrolysate of phosphatides was carried out by the method of Magee *et al.* (16).

The authentic samples of phosphatidylethanolamine, phosphatidylserine and inositol phosphatide were prepared by the method of Folch (17).

RESULTS

Analysis by Column Chromatography—The radioactivity and total P of each fraction of heart and femoral muscle phosphatides at 2 and 17 hours after the intraperitoneal injection of P³² are shown in Fig. 1. Fig. 2 shows the radioautograms obtained by the paper

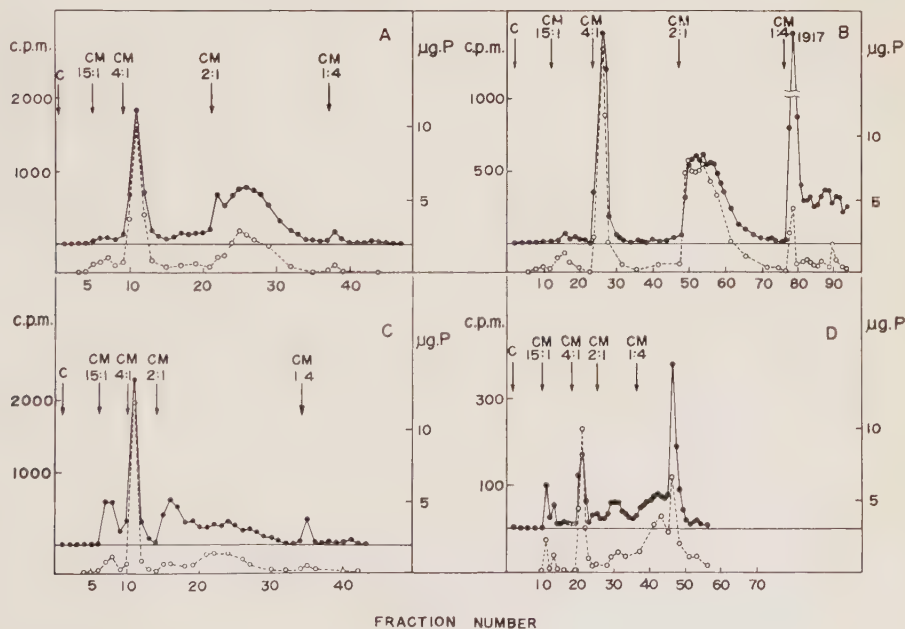


FIG. 1. Column chromatography of rat heart and femoral muscle phosphatides.

A, B and C, D represent heart and femoral muscle phosphatides respectively, and 16 hours after P³² injection for A and C and 2 hours for B and D. The heart and femoral muscle used were from the same animal. The solid lines are elution curves of radioactivity and the dotted lines are lipid P. Each fraction in A and B is 10 ml., and in C and D 5 ml. CM=chloroform-methanol.

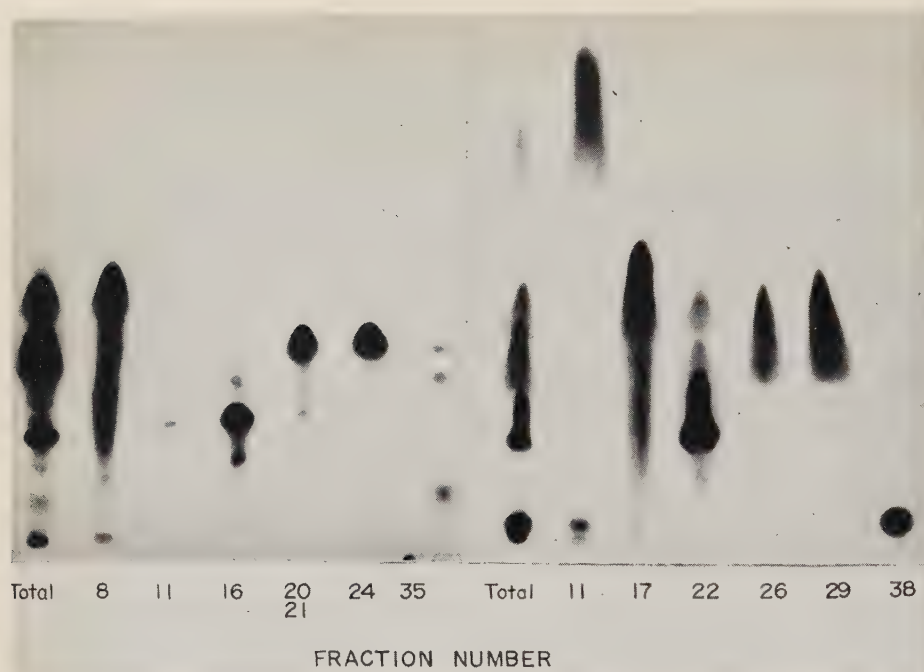


FIG. 2. Radioautograms of P^{32} -labelled rat heart phosphatides in each fractions in Fig. 1A and B.

chromatography of each fraction on silicic acid impregnated paper shown in Fig. 1A, and Fig. 3 shows the tracings of the paper chromatograms of each fraction shown in Fig. 1. The percentage distribution of radioactivity and the results of detection by rhodamine 6 G, ninhydrin and choline reactions are also shown in Fig. 3.

The first peak of heart muscle phosphatides eluted from silicic acid column with chloroform-methanol 15:1, was mainly phosphatidic acid (Fig. 3). At the 2nd hour after the P^{32} injection, this fraction showed a higher specific radioactivity than that at the 16th hour. The next sharp peak eluted with chloroform-methanol 4:1 was mainly phosphatidylethanolamine. In many cases, this spot showed long tailing on the silicic acid impregnated paper. In hydrolysate of this fraction, however, serin was not detected, almost all the N-compound being ethanolamine. The specific radioactivity of this fraction was higher at the 16th hour than at the 2nd hour of the injection.

In the next peaks eluted with chloroform-methanol 2:1, were found 2 components, of which the first one, some times overlapped with the second, contained more than 80 per cent of its radioactivity in the form of inositolphosphatide, as shown in Fig. 3. This fraction showed the highest specific radioactivity of all the phosphatide fractions. The next rather flat and tailed peak included mainly lecithin. The following smaller peaks were sphingomyelin mixed with orthophosphate, and probably lysolecithin in this sequence. The specific radioactivity of each fraction as a whole is shown under each figure in Fig. 3. However these values do not correspond to the specific radioactivity of each phosphatide component, since each fraction does not necessarily consist of a single phosphatide. It was impossible to obtain the specific radioactivity of each phosphatide fraction, because the analytical values of total P of each spot were not constant. This might be due to the inhibitory effect of silicic acid on the color development

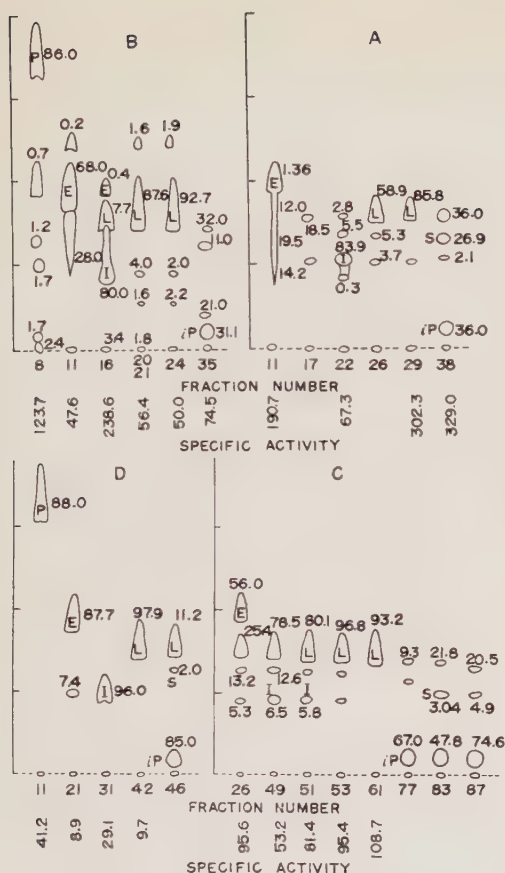


FIG. 3. Tracing of a chromatogram of P^{32} -labelled rat heart phosphatides in each fraction in Fig. 1A and B.

The numbers by each spot refer to the percentage of each spot to the total radioactivity of each fraction and the numbers under the fraction number refer to the specific radioactivity of each fraction. L=lecithin, stained yellow with Rhodamine 6 G, choline test was positive with phosphomolybdate- $SnCl_2$. E=phosphatidylethanolamine, ninhydrin positive, yellow with Rhodamine 6 G. I=inositol phosphatide, purple with Rhodamine 6 G. S=spingomyelin, yellow with Rhodamine 6 G, choline test was weakly positive. IP=inorganic phosphate.

of phosphomolybdic acid and the loss, during treatment, of a part of silicic acid powder, which adsorbed phosphatides.

The specific radioactivity of each fraction of femoral muscle phosphatides was remark-

ably lower than that of heart muscle. An appreciable quantity of P^{32} was incorporated into phosphatidic acid of femoral muscle after 2 hours, but not after 17 hours as in the case of heart muscle (Fig. 2b).

Inositolphosphatide showed the highest specific radioactivity in femoral muscle, but the value was lower than that in heart muscle. Only in the case of femoral muscle, a large amount of orthophosphate was eluted together with spingomyelin.

The specific radioactivity of total phosphatides of heart muscle was about 8 times as high as that of femoral muscle at the 2nd hour after P^{32} injection, but at the 16th hour both types of muscle showed almost the same activity. The result suggests that the turnover rate of heart muscle phosphatides was higher than that of femoral muscle.

Analysis of Muscle Phosphatide Fraction by the Paper Chromatography of its Mild Alkaline Hydrolysate— Fig. 4 shows the radioautograms obtained by the paper chromatography of the mild alkaline hydrolysates of heart and femoral muscle phosphatide fractions. Total phosphate and radioactivity were measured only in the extracts of the spots, which were identified exactly, *e.g.* in those of glycerylphosphorylcholine, glycerylphosphorylethanolamine, inositol phosphatide and glycerophosphoric acid. The phosphatide P of heart and femoral muscle was 500–750 and 300–350 μ g. per g. of fresh tissue respectively, and the relative content of P in each phosphatide fraction was shown in percentage in Table I. In both types of muscle, glycerylphosphorylcholine was the most abundant phosphatide, being greater in femoral muscle, and phosphatidylethanolamine came next.

As shown in Fig. 5 (A and C), incorporation of P^{32} was highest in glycerylphosphorylcholine fraction in both muscles, being higher in femoral muscle, where 40–50% of the total radioactivity incorporated into the phosphatides was present in this fraction.

The relative specific activities $\frac{\text{c. p. m. of an individual phosphatide}}{\text{c. p. m. of the total phosphatides}}$ μ g. P of an μ g. P of the total

TABLE I
Content of Phosphatides in Muscle

Phosphatides	No. of experiments	P in per cent of total phosphatide P	
		Heart muscle	Femoral muscle
Glycerylphosphorylcholine	6	32.4±2.5	46.4±6.4
Glycerylphosphorylethanolamine	6	18.7±6.2	21.5±2.5
Glycerophosphoric acid	6	8.5±4.8	5.1±1.7
Hydrolysis product of inositol phosphatide	6	10.7±4.0	7.8±1.6

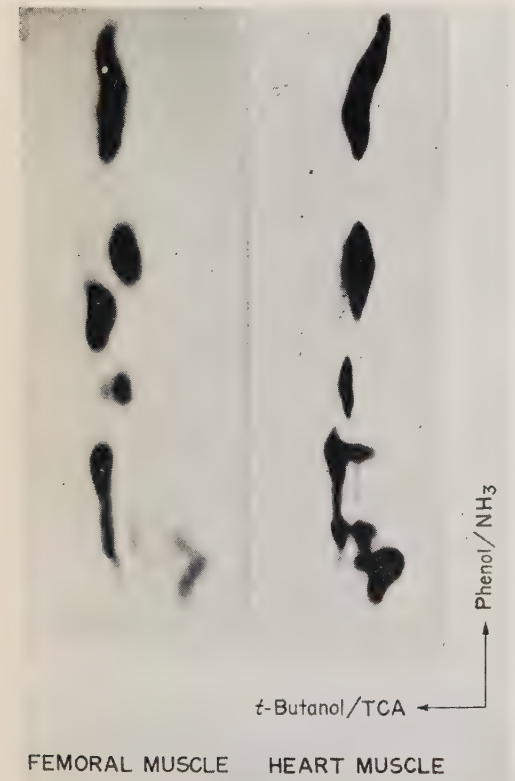


FIG. 4. Two-dimensional radioautograms of P^{32} -labelled heart and femoral muscle phosphatide fraction.

individual phosphatide $\times 100$, at various periods of time after P^{32} injection were shown in B and D of Fig. 5. It is striking that the relative specific activity of inositol phosphatide in the heart muscle was remarkably high and that of phosphatidic acid was high only for a short time after the

injection, as in the case of column chromatography with silicic acid (Fig. 1). The same results were obtained in femoral muscle, but the values were lower than those in heart muscle. The relative specific activity of glycerylphosphorylcholine in both muscles reached 100 per cent sooner or later after the injection of P^{32} , but that of glycerylphos-

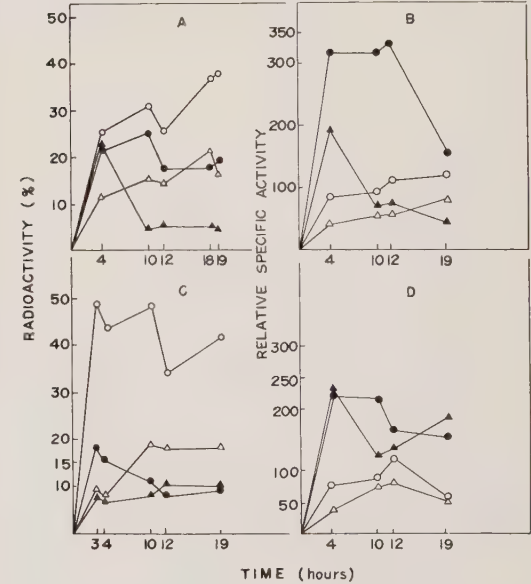


FIG. 5. Per cent of radioactivity of total phosphatides and specific radioactivity in each phosphatides on the paper chromatogram carried out by Dawson's method. Ordinate shows the times after P^{32} injection. —○—, glycerylphosphorylcholine; —●—, hydrolysis products of inositol phosphatides; —△—, glycerylphosphorylethanolamine; —▲—, glycerophosphoric acid. A, B and C, D show the heart and femoral muscle phosphatides respectively.

phorylethanolamine did not reach 100 per cent even at 19 hours after the injection. To reach 100 per cent means that specific radioactivity of an individual phosphatide is equal to that of the total phosphatide at that time.

Analysis of Muscle Phosphatides by Means of Silicic Acid Impregnated Paper—The percentage distribution of radioactivities of the muscle phosphatides at various times up to 19 hours after P^{32} injection, analysed by chromatography on silicic acid impregnated paper, was shown in Fig. 6. Incorporation of P^{32}

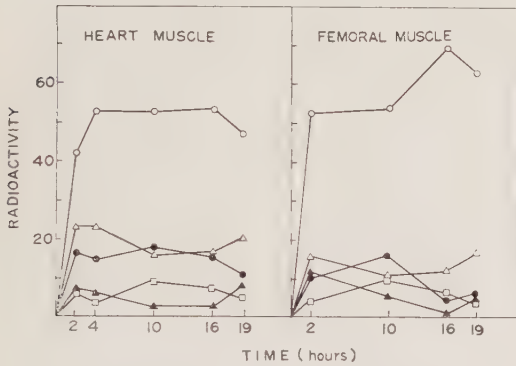


FIG. 6. Per cent of radioactivity of total phosphatides in each phosphatides on silicic acid impregnated paper.

Ordinate shows the times after P^{32} injection.

—○—, lecithin; —●—, inositol phosphatide; —△—, phosphatidylethanolamine; —▲—, phosphatidic acid; —□—, sphingomyelin.

into lecithin spot of both heart and femoral muscle phosphatides were greater than that into glycerylphosphorylcholine shown in Fig. 5 (A and C). This discrepancy in the percentage incorporation of P^{32} into lecithin between both paper chromatographic analyses, suggests that the lecithin spot on the silicic acid impregnated paper contains more than one phosphatide.

Rechromatography by the method of Dawson of mild alkaline hydrolysates of the elutes obtained from the central portion of each spot on the silicic acid impregnated paper (Fig. 7A, B and C), showed that each spot on the silicic acid impregnated paper consisted of more than one component. (Fig.

8).

These results can account for the discrepancy in the values of percentage distribution of P^{32} between lecithin and glycerylphosphorylcholine spots in both paper chromatographic techniques.

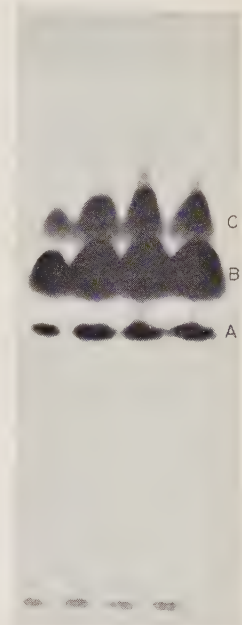


FIG. 7. Radioautogram of P^{32} -labelled rat heart phosphatides.

Paper chromatography was carried out on silicic acid impregnated paper. A=inositol phosphatide, B=lecithin, C=phosphatidylethanolamine. About 30 μ g. P of each phosphatide was pipetted on the each base line.

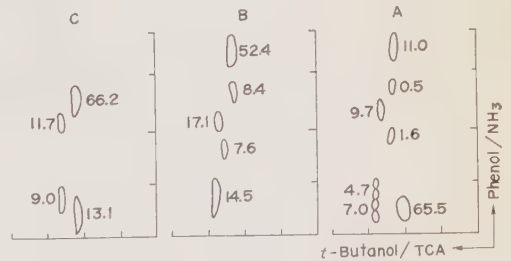


FIG. 8. Rechromatography of the elutes from paper chromatographic single spot.

A, B and C represent the hydrolysis products of spot A, B and C in Fig. 7. The numbers by each spot refer to the per cent of total radioactivity in each eluate.

More than 90 per cent of radioactivity of the hydrolysate of the eluate from the lecithin spot on Marinetti's paper chromatogram, which was obtained using the lecithin fraction separated by column chromatography, was found in glycerylphosphorylcholine.

DISCUSSION

The quantitative separation of tissue phosphatides is an essential process for the studies of the metabolism of tissue phosphatides. Three methods described in this paper, which are generally used, however, are unsatisfactory for the quantitative separation of phosphatides, so far as each of them is used alone.

The chromatography on silicic acid impregnated paper has shown itself to be unsuitable for the quantitative determination of phosphatides, even when the lipids containing 30 μ g. of phosphatide P were applied, because each spot separated on the paper did not reveal a single phosphatide. But the method is useful in examining the composition of each fraction separated by the silicic acid column chromatography. In this method, however, it was impossible to estimate the phosphatide P exactly, since a part of silicic acid powder which adsorbed phosphatides might be lost during the process of determination, and the content of phosphate in certain spots was too small for accurate determination. Each spot on the paper chromatogram of mild alkaline hydrolysate may correspond to each of the hydrolysis products of phosphatides, but the formation of artifacts could not be excluded. Although the separation of phosphatide was not perfect by column chromatography on silicic acid, the result obtained by this method was more reproducible than by the other two methods, and it also made possible the separation of large amounts of phosphatides.

To estimate the phosphatide content of the tissue at the present state, therefore, it seems to be necessary to apply more than two techniques at the same time.

Since heart muscle is more active than

femoral muscle and the turnover of phosphatide P in the former is higher than in the latter, a part of the phosphatides might be utilized as endogenous substrate or as intermediate of lipid utilization in heart muscle.

Inositolphosphatide, in spite of its small content, has the highest specific radioactivity in muscle, particularly in heart muscle. Since similar results have been reported in the case of brain (7), avian salt gland (9), and pancreas (7), this phosphatide might play an important role in certain common mechanism or mechanisms.

High specific radioactivity of phosphatidic acid only for a short time after P^{32} injection suggests that this phosphatide may be an intermediate of *in vivo* synthesis of other phosphatides, as already indicated by Kennedy (18). In spite of its relatively large content in muscle, phosphatidylethanolamine has the lowest specific activity, suggesting that this phosphatide may exist as a structural substance in some part of the cell.

SUMMARY

The incorporation of P^{32} -labelled orthophosphate into heart and femoral muscle phosphatides of rat has been investigated *in vivo* by three methods: column chromatography with silicic acid, paper chromatography on silicic acid-impregnated paper and two-dimensional paper chromatography of hydrolysates of phosphatides. From the results obtained some criticism of those methods was made.

1. The phosphatide content of heart muscle (500–750 μ g. of P per g. of wet tissue) was about twice as great as in femoral muscle (300–350 μ g. of P per g. tissue). The specific radioactivity of phosphatides in heart muscle, was about 7–8 times as high as that in femoral muscle at the second hour after the injection of P^{32} . However, these muscles showed almost the same activity 16 hours after the injection.

2. Lecithin was the most abundant phosphatide, and phosphatidylethanolamine was the next in both muscles, and the sum of the two phosphatides amounted to more

than 51 and 68 per cent of the total phosphatides in heart and in femoral muscle respectively.

3. The radioactivity of P³² incorporated into lecithin was about 50 per cent of the radioactivity incorporated into the total phosphatides in both types of muscle. However the specific radioactivity of lecithin was lower than that of the total phosphatides until 16 hours after P³² injection.

4. The specific radioactivity of inositol phosphatide was highest, particularly in heart muscle. It was about 3 times as high as that of the total phosphatides.

5. The specific radioactivity of phosphatidic acid was high only at the early stage after P³² injection.

6. The specific radioactivity of phosphatidylethanolamine was lowest, in spite of its relatively large content in the tissue.

7. A single spot separated on silicic acid impregnated paper did not consist of a single phosphatide, at least when an exactly measurable amount of phosphatides was applied. Therefore this technique by itself seemed to be insufficient for quantitative analysis of phosphatides. It was useful, however, in determining the composition of each fraction separated previously by column chromatography with silicic acid.

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Formation of Thyroglobulin-bound Iodine in Cell-free System and Effect of Thyrotropic Hormone*

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In the course of studies on the action of thyrotropic hormone (TSH), various aspects of iodine metabolism in the presence or absence of TSH, have been investigated by employing tissue slices of pig thyroid. In these studies (2-3), it has been demonstrated that TSH definitely enhances the conversion of iodide to thyroglobulin-bound iodine, *in vitro*. It was thought, therefore, necessary to investigate the iodination reaction of thyroglobulin in more simplified reaction system than tissue slices and to check the effect of TSH.

The synthesis of organic iodine in cell-free system has been demonstrated by several investigators. However, the reported products were iodotyrosines (4-8), iodinated serum albumin (8) or iodinated particulate fraction (9-12), and the iodination of thyroglobulin has not been demonstrated. Physiological significance of these systems is, therefore, not clear. No effect of TSH upon the reaction has been reported.

The purpose of the present investigation is to reconstruct a cell-free system which is capable of iodinating thyroglobulin under physiological condition. The cell-free system described in this paper showed an effective capacity for the iodination when supplemented with an H_2O_2 -generating system. It was also observed that TSH brought about effects upon the reaction in this system.

EXPERIMENTALS

Preparation of Subcellular Particulate Fractions—Pig thyroid glands were obtained at a slaughter-house and

kept on ice until used. Within 2 hours after the animals were killed, following preparations were initiated. All operations were conducted at 0-5°C.

After removal of extraneous tissues, five to ten glands were minced with a razor blade, and homogenized in an equal volume of 0.25 *M* sucrose solution containing 0.002 *M* ethylenediaminetetraacetate (EDTA). Homogenization was performed in a blender for 30 seconds at 20 volts and for 10 seconds at 100 volts. The homogenate was filtered through layered gauze and diluted with the homogenization medium to make 10% homogenate.

The homogenate was centrifuged in a refrigerated centrifuge at $700 \times g$ for 10 minutes and the sedimented materials were discarded. From the supernatant, the mitochondrial fraction was sedimented by means of centrifugation at $5,000 \times g$ for 10 minutes. The pellet was resuspended in a half volume of the supernatant at $700 \times g$ and centrifuged again. The sedimented mitochondria were suspended in an appropriate volume of 0.25 *M* sucrose solution containing 0.002 *M* EDTA. This fraction was designated as S_5 .

The supernatant obtained in the first centrifugation at $5,000 \times g$ was centrifuged at $80,000 \times g$ for 30 minutes. For this purpose a Hitachi preparative ultracentrifuge (Rotor: No. 40) was used. After the inner wall of the centrifuge tube was carefully wiped with filter paper, the firmly packed pellet was gently homogenized in an appropriate volume of the sucrose solution containing EDTA. This fraction was designated as S_{5-80} .

The mitochondrial-microsomal fraction, S_{80} , was obtained by directly centrifuging the $700 \times g$ supernatant at $80,000 \times g$ for 30 minutes. The firmly packed pellet thus obtained was resuspended in an appropriate volume of the sucrose solution. The color of the pellet varied with preparations from yellowish red to reddish yellow.

Preparation of Thyroglobulin—The preparation of thyroglobulin chiefly used in this study was prepared according to a modified method (13) of Derrien *et al.* (14) from frozen pig thyroid glands. It was almost

* A part of this work has been reported in the preliminary report (1), and was read before the 33rd annual meeting of the Japanese Biochemical Society.

homogeneous on the basis of ultracentrifugal examination.

A thyroglobulin fraction with low iodine content used in a few experiments was obtained from the above preparation by DEAE-cellulose chromatography according to the method of *Ui et al.* (15). It was ultracentrifugally homogeneous.

Other Materials—Glucose oxidase was purchased from Nutritional Biochemicals Corp., U. S. A. Crystalline serum and egg albumines were prepared according to the Kekwick's method (16, 17).

TSH was prepared in this laboratory* from frozen whale pituitaries according to the method essentially similar to that of Ciereszko (18). Its activity was approximately 1 U.S.P. unit per mg.

Incubation—The standard incubation system contained 0.2 ml. of pig thyroid mitochondrial-microsomal fraction (S_{80}), approximately 15 mg. of purified thyroglobulin, 0.08 μ moles of KI, 10 μ c of carrier-free NaI^{131} , 140 μ moles of NaCl, 10 μ moles of phosphate buffer (pH 7.2), and 3 μ moles of glucose and 15 μ g. of glucose oxidase, as an H_2O_2 -generating system, all in a total volume of 1.0 ml. A 20 ml. flask containing the reaction mixture was incubated at 37°C in a shaking incubator under the air.

Preceding to the initiation of the reaction, the flasks containing all the constituents except glucose oxidase were pre-incubated for a few minutes to attain the temperature equilibrium. Then, the reaction was started with adding glucose oxidase. During the incubation, 0.1 ml. of the reaction mixture was pipetted out at intervals and mixed with 0.01 ml. of 5% thio-urea to stop the reaction.

In studies in which effect of TSH was examined, 100 μ g. of TSH preparation was added to the standard incubation mixture.

Analyses of the Reaction Mixture—An aliquot (0.01–0.02 ml.) of the incubated mixture mentioned above was subjected to paper electrophoresis in order to separate thyroglobulin from other cellular components and iodide. Electrophoresis was performed for 5 to 10 hours at a constant current density of 0.45 mA/cm. in a veronal buffer of pH 8.6 and ionic strength of 0.075. Thyroglobulin and other components on the paper were located by the radioautogram after exposure of the paper to Fuji X-ray film for an appropriate period. For quantitative estimation of the labeled components, the paper was cut out and counted in a well-type scintillation counter.

If necessary, paper strips were stained with bromophenol blue.

For the preparation of labeled thyroglobulin and labeled particulates, 10 ml. of the standard incubation mixture was incubated for 15 minutes at 37°C. After stopping the reaction, the mixture was centrifuged at 80,000 $\times g$ for 30 minutes to separate the particulates. The precipitate, after being purified by the repeated centrifugations, was suspended in a Krebs-Ringer phosphate buffer (pH 7.2). Labeled thyroglobulin was purified from the 80,000 $\times g$ supernatant by means of salting-out with ammonium sulfate. The formed precipitate was dissolved and dialyzed against Krebs-Ringer phosphate buffer (pH 7.2). This solution or suspension was counted in a well-type counter.

Chromatographic Identification of Iodinated Substances—The solution of labeled thyroglobulin and the suspension of labeled particulates were digested by "Pronase" (*Streptomyces protease*) at pH 7.2. Each 0.02 ml. of the digests was chromatographed using *n*-butanol-acetic acid-water (78:5:17, in v:v:v) as mobile phase. The paper strips obtained were treated in the same manner as in the case of paper electrophoresis.

RESULTS

Essential Constituents in the Iodination Reaction—When purified pig thyroglobulin was

TABLE I

The Requirement of H_2O_2 -Generating System and Thyroid Subcellular Particulate Fraction in the Incorporation of I^{131} -Iodide into Thyroglobulin

System	% of I^{131} activity incorporated into thyroglobulin
Complete system	4.29
Complete minus particulate fraction S_{80}	0.01
Complete minus glucose oxidase	0.01
Complete minus glucose	0.03
Complete, with heated S_{80}	0.01
Complete, with liver particulate fraction instead of thyroid preparation S_{80}	0.01

The complete system, as described in text, contained thyroid particulate fraction S_{80} , purified thyroglobulin, KI labeled with I^{131} , glucose and glucose oxidase as an H_2O_2 -generating system, all in a total volume of 1.0 ml. Incubations were carried out for 5 minutes at pH 7.2 and 37°C, under shaking in the air. Heated S_{80} was prepared by heating the fresh preparation at 100°C for 5 minutes. Liver particulate fraction was prepared following the same procedure as the preparative procedure of thyroid S_{80} fraction.

* The method to be published.

aerobically incubated with I^{131} in the standard incubation system at 37°C for 5 minutes, I^{131} was found to be incorporated into thyroglobulin. The results are shown in Table I, and the paper electrophoretic diagram of the incubated mixture is reproduced in Fig. 1. Thyroglobulin was not labeled when any of the constituents (the particulate fraction, glucose, glucose oxidase) was omitted from the system, or when the particulates were replaced with boiled ones. Liver subcellular fraction could not replace thyroidal particulate fraction (Table I). These findings

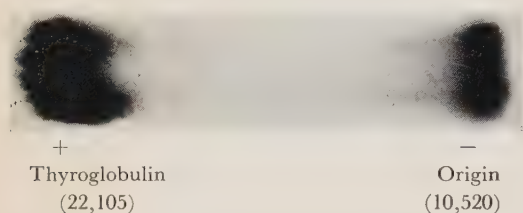


FIG. 1. Radioautogram of paper electrophoretic diagram of the labeled proteins after incubation in the standard incubation system for 5 minutes.

Number enclosed in bracket expresses radioactivity found in the area (c.p.m.).

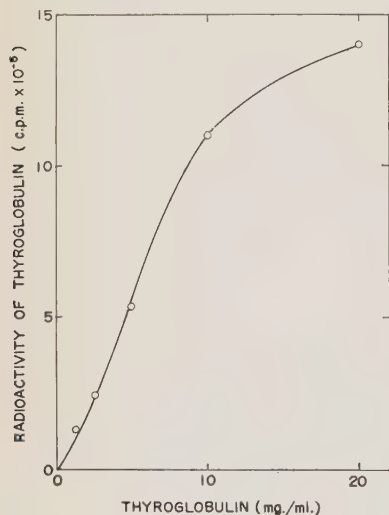


FIG. 2. Effect of concentration of thyroglobulin on the iodination of thyroglobulin.

Incubations were performed in the standard system except that the concentration of thyroglobulin was varied as shown in abscissa.

Incubation: 60 minutes, at 37°C . Concentration of particulate fraction: 0.8 mg. N/ml.

show the requirements of the thyroid particulate fraction and the H_2O_2 -generating system for the labeling of thyroglobulin.

The labeling was found to be proportional to the concentration of thyroglobulin, at least, up to 1.0 per cent (Fig. 2).

The relation between the extent of labeling of thyroglobulin and the concentration of particulate fraction was not so simple. The optimal concentration varied from 0.1 to 0.3 mg. protein N per ml., with experiments. The labeling decreased with either increase or decrease in the concentration of particulates from the optimal concentration. This is illustrated in Fig. 3.

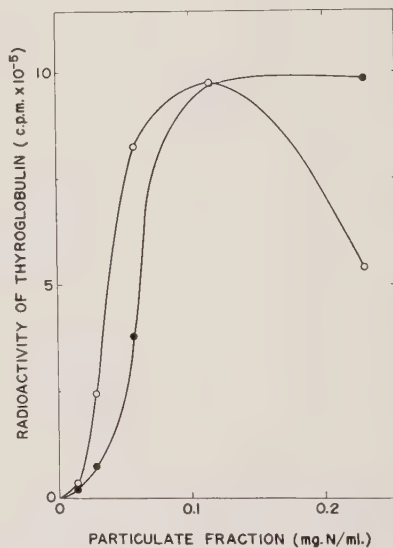


FIG. 3. Effect of concentration of thyroid particulates fraction upon the rate of I^{131} incorporation into thyroglobulin.

Open circle: standard system. Solid circle: standard system with S_{5-80} instead of S_{80} .

Concentration of S_{80} is shown in abscissa. Concentration of S_{5-80} is expressed by the concentration of S_{80} prepared from the same amount of homogenate. Incubation: 5 minutes.

At the concentration below $75 \mu\text{g./ml.}$, the incorporation depended on the concentration of glucose oxidase, but further incorporation was not observed even at higher concentration than $75 \mu\text{g./ml.}$ Iodide concentration in the reaction mixture also affected the

extent of the reaction (Fig. 4).

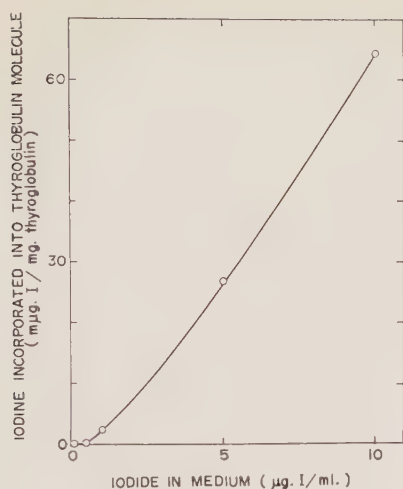


FIG. 4. Effect of concentration of iodide in medium upon the iodination of thyroglobulin.

Incubation were performed for 5 minutes in the standard system except that the concentration of iodide in medium was varied as shown in abscissa. The amount of iodide incorporated into thyroglobulin molecule expressed in ordinate was calculated from per cent I^{131} activity incorporated into thyroglobulin and specific activity of iodide in medium. Particulate fraction: 0.2 mg. N/ml.

Role of Various Cellular Fractions—The reaction systems, in which particulate fraction S_{5-80} was used instead of S_{80} , was compared with the standard system over wide range of particulate concentration. The typical results are included in Fig. 3. The same extent of labeling was observed in the both cases when the concentration of particulates was optimal. However, at the concentrations lower than optimal, the labeling of thyroglobulin by S_{5-80} lowered more steeply with the decreasing particulate concentration than that by S_{80} . The sole addition of S_5 did not proceed the labeling of thyroglobulin (Table II).

Nuclear fraction was found to have no effect. When whole homogenate was used instead of particulate fraction, iodination scarcely occurred (Table II). This finding might be explained from the proposed inhibitor present in the supernatant (8).

TABLE II

Iodinating Activity of Thyroid Cellular Preparation

Expt. No.	Cellular preparation	I^{131} incorporation into thyroglobulin c.p.m. $\times 10^{-3}$
1	Homogenate	7.9
	Particulate fraction S_{80}	412
	Particulate fraction (S_{80}) ₁	550
2	Particulate fraction S_{80}	553
	Particulate fraction S_{5-80}	250
	Particulate fraction S_5	6.0

Incubations were carried out in the standard incubation system in which S_{80} fraction was replaced with various cellular preparations. (S_{80}) was obtained by centrifuging the homogenate at $80,000 \times g$ for 30 minutes. Concentration of particulate preparation; S_{80} in Expt. 1, 0.30 mg. N/ml.; S_{80} in Expt. 2, 0.23 mg. N/ml. Other preparations were prepared from the same amount of homogenate from which S_{80} was prepared. Incubation time: 5 minutes.

Time Course—The iodination in the complete system increased with time, at least for 15 minutes. The typical time course is shown in Fig. 5. In the first few minutes, the slope of the curve was gentler than that in the

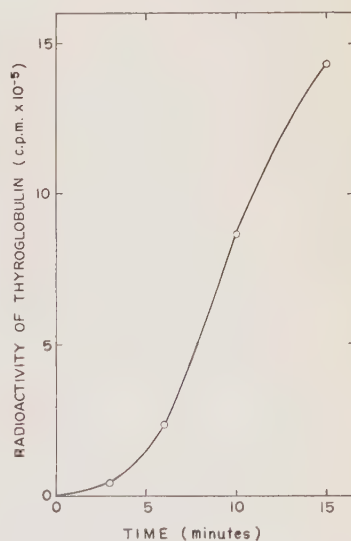


FIG. 5. Time course of the iodination of thyroglobulin in the standard incubation system.

Concentration of particulate fraction: 0.22 mg. N/ml.

later period. The reason for this phenomenon is not clear.

Incorporation I^{131} into Particulates—During the incubation in the standard system, considerable incorporation of I^{131} was found to occur into the particulate fraction, too. This can be shown by the radioactivity remained at the origin of paper electrophoresis (see Fig. 1), or by the radioactivity of the particulates recovered from the incubated reaction mixture by centrifugation.

The ratio of I^{131} activities in thyroglobulin and in the particulates fraction varied with the concentration ratio of thyroglobulin to particulates in the reaction mixture. In optimal conditions, the I^{131} ratio reached to 1.5 to 2.0. (Fig. 1).

In order to determine whether the labeled particulate protein is a precursor of labeled thyroglobulin or not, the separated labeled particulates were incubated with non-labeled thyroglobulin in the standard system without containing NaI^{131} . In this case, no iodination of thyroglobulin was found to occur. On the other hand, pre-labeled thyroglobulin, too, failed to cause the labeling of the particulate fraction. Thus, these two iodination reactions, *i. e.*, iodination of thyroglobulin and iodination of particulates, were suggested to be independent.

Stability of Thyroglobulin-bound I^{131} —In order to examine the possibility that the incorporation might occur by an exchange reaction, the following experiment was performed. The labeled thyroglobulin, obtained by incubation in the standard system, was incubated with stable iodide in the same system without containing NaI^{131} . By this incubation, neither radioactive iodide nor labeled iodinated amino acids were found to appear in the medium. The result suggests that the labeling of thyroglobulin is caused by a true iodinating reaction, and that no hydrolytic breakdown of thyroglobulin occurs in the present system.

Localization of I^{131} in Thyroglobulin and Particulates—Radioautograms of paper strips obtained after chromatography of the hydrolysates indicated that the I^{131} in thyroglobulin is localized almost exclusively in moniodo-

tyrosine and diiodotyrosine (Fig. 6 (A)). A radioactive spot, found in the area of R_f 0.2, was thought to be due to iodide produced by deiodination of iodinated amino acids during enzymatic digestion.

I^{131} in the digest of the labeled particulate fraction was found to exist as iodide, moniodotyrosine, diiodotyrosine and iodinated lipid, with R_f of nearly 1 (β) (Fig. 6 (B)). It is likely that a large portion of labeled iodide found in this case is due to the contamination from the medium.



FIG. 6. Radioautogram of paper chromatogram of thyroglobulin hydrolysate (A) and hydrolysate of particulate fraction (B).

Chromatographic solvent: *n*-butanol-acetic acid-water (78:5:17). MIT: moniodotyrosine. DIT: diiodotyrosine. L: iodinated lipid.

Specificity of Thyroglobulin as Substrate—When serum or egg albumin, instead of thyroglobulin, was added to the standard incubation mixture at the same concentration, the extent of iodination was very low even after 10 minutes' incubation. The results are shown

in Fig. 7.

It was also found that chromatographically fractionated thyroglobulin with low iodine content (15) was more easily iodinated than unfractionated thyroglobulin (Table III).

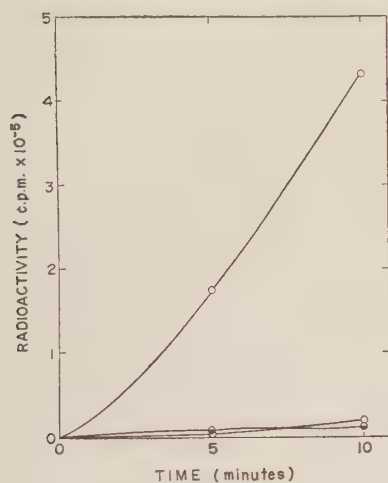


FIG. 7. Specificity of thyroglobulin as substrate in the iodination system.

The three reaction systems contained as substrate protein, 12mg. of thyroglobulin (open circle), serum albumin (solid circle) and egg albumin (semi-solid circle), respectively. Concentration of particulate fraction: 0.45 mg. N/ml. in all reaction systems.

TABLE III

Chromatographically Fractionated Thyroglobulin as Substrate in the Iodination Reaction

Thyroglobulin preparation	I^{131} incorporation into thyroglobulin c.p.m. $\times 10^{-3}$
Chromatographically fractionated preparation	458
Unfractionated preparation	55

Incubations were performed in the standard incubation system at 37°C for 5 minutes. Concentration of particulate fraction was 0.44 mg. N/ml.

Effect of Magnesium or Calcium Ions—Incorporation of I^{131} -iodine was strongly inhibited by the addition of Mg^{++} or Ca^{++} as shown in Fig. 8.

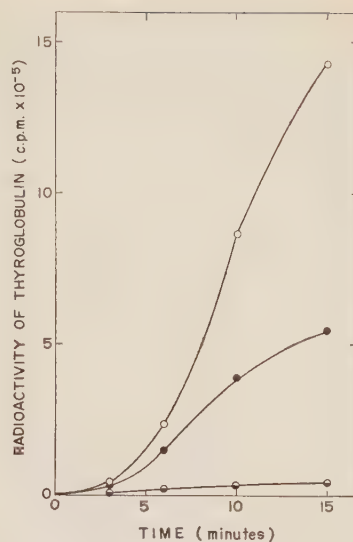


FIG. 8. Inhibitory effect of magnesium or calcium ion upon the iodination of thyroglobulin.

Open circle: control. Solid circle: control plus $1.5 \times 10^{-3} M Mg^{++}$. Semi-solid circle: control plus $1.5 \times 10^{-3} M Mg^{++}$ and $2.3 \times 10^{-3} M Ca^{++}$. Concentration of particulate fraction: 0.22 mg. N/ml.

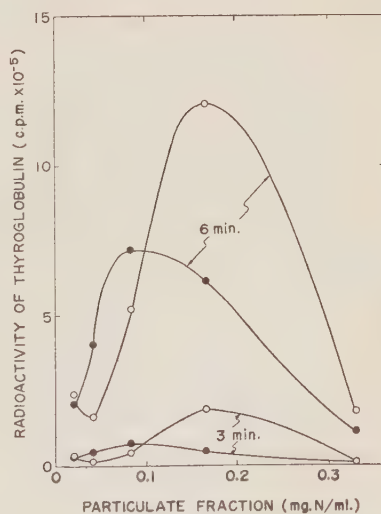


FIG. 9. Effect of thyrotropic hormone upon the iodination of thyroglobulin.

Incubations were performed in the standard system except in the case of the concentration of particulate fraction which was varied as shown in abscissa. Incubation time is shown in the figure.

It was found that the effect of Mg^{++} or Ca^{++} ions was not mediated by inhibition of the H_2O_2 -generating reaction. When glucose and glucose oxidase were incubated in a Warburg manometer in the presence or absence of these cations, no change was observed in O_2 -uptake.

It was also observed in this laboratory that peroxidase activity of particulate fraction S_{80} was, if measured by the guaiacol method, not influenced by the addition of Mg^{++} or Ca^{++} (19).

Effect of TSH—When the incubation was performed in the presence of 100 μg . of TSH preparation, various effects were observed upon the I^{131} incorporation. Fig. 9 shows that the iodination of thyroglobulin is either stimulated or inhibited according to the concentration of particulate fraction.

DISCUSSION

The essentiality of the H_2O_2 -generating system in the present cell-free iodinating system agrees with the finding of Alexander who demonstrated the iodination of tyrosine by thyroid homogenates upon supplementation with glucose and glucose oxidase (7). In the present system, the concentrations of glucose and glucose oxidase were decided from experiments as the minimum concentration to show full capacity for the iodination. The data shown in Table I suggest that the generated H_2O_2 (at a rate of 0.1 $\mu moles$ per minute) does not iodinate substrates non-enzymatically. The high concentration of thyroglobulin (1.5 per cent) in the standard incubation mixture might give rather strange impression, but it should be remembered that the concentration of thyroglobulin in thyroid tissue is usually 0.05–0.1 g. per 1 g. of wet tissue*, which corresponds to 5–10% solution. In the standard system, the concentration of thyroglobulin was fixed to 1.5 per cent for the sake of experimental convenience. The concentration of iodide (0.08 $\mu moles$ per ml. as KI) roughly corresponds to its concentration in pig thyroid glands, *i. e.*, 0.1–1.0 μg . per

100 mg. of wet tissue (2). The dependency of the extent of iodination upon the concentration of particulates is notable and might suggest the complexity of the reactions involved.

Remarkable is the finding of the specificity of the iodination reaction in the present system. The iodination was found to occur in thyroglobulin, but not in egg albumin nor in serum albumin. In the case of a fractionated thyroglobulin with low iodine content, the iodination proceeded more easily. Recently, Suzuki, Nagashima and Yamamoto (8) reported that the labeling of serum albumin occurred by their tyrosine-iodinating system in which thyroidal particulates were used instead of whole homogenate in Alexander's system. The reason for this discrepancy is not clear, but might be due to the difference in incubation time as well as other experimental conditions.

Incorporation of I^{131} into particulate protein by cell-free system has been reported by several investigators (9–12). Although it also occurred in this study, the extent of incorporation into particulates was definitely less than that into thyroglobulin in the present system. Attempts to find out the relation between these two iodination reactions were unsuccessful. It might be probable that the iodinated particulates are non-specific by-products, but further work would be necessary to confirm this.

Since Mg^{++} or Ca^{++} ions do not affect either glucose oxidase or peroxidase activity, the inhibition of the iodination reaction caused by these cations would be probably due either to the effects on other iodinating mechanism involved in the particulate fraction, or to those on the integrity of the particulate structure. If it is taken into consideration that the partially purified preparation of peroxidase, solubilized from thyroidal microsomes (19), could replace the fraction S_{5-80} in the present iodination reaction, the former view seems to be preferable. Investigation for the Mg^{++} - and Ca^{++} -sensitive reaction might be valuable for further elucidation of the iodination mechanism in thyroid gland. Alexander assumed the presence of an

* Unpublished data.

enzyme (tyrosine iodinase) which catalyzes the transfer of "active" iodine to tyrosine (7).

Effect of TSH in the present cell-free system is of special interest since there have been few studies on the hormonal effect at a cell-free level. However, many questions are remained to be elucidated. Although scattered results on the effects of TSH obtained in the preliminary experiments were partially explained from the results shown in Fig. 9, it is not clear, at present, whether these effects of TSH are physiological or not. Further studies on this point are now in progress and will be reported in near future.

In conclusion, it should be emphasized that the present thyroidal cell-free system is able to iodinate thyroglobulin rather efficiently under mild condition and has some features which are similar to the iodinating mechanism of intact thyroids. It might be expected that investigations using the present system or modified ones provide information about mechanism of intrathyroidal iodination and the regulation caused by TSH.

SUMMARY

A thyroidal cell-free system which is able to iodinate thyroglobulin effectively is reported.

When thyroglobulin was aerobically incubated at 37°C with pig thyroid particulate fraction (mitochondria-microsomes), iodide, glucose and glucose oxidase, iodine was incorporated into thyroglobulin efficiently, and iodinated amino acids were formed. The particulate fraction, too, was iodinated, but the extent was less than in the case of thyroglobulin. The reaction appears to be specific since the liver particulates fraction was ineffective and serum or egg albumin could not be iodinated in the present system. Mg^{++}

or Ca^{++} ions inhibited the reaction. Thyrotropic hormone (TSH) was found to affect the reaction, but the effect depended upon the concentration of particulates in the incubation medium.

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A Model for the Myosin ATPase Active Site*

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The adenosine triphosphatase (ATPase) of myosin, which was first reported by Engelhardt and Ljubimova (1, 2), has been the subject of many investigators (3-6) of muscle proteins, since it is probably closely related to muscle contraction.

The general characteristics of this enzyme have become known through the efforts of many workers, although there are some controversies due to the complexity of the system. Direct approach to the active site and enzymatic mechanism has been pursued using a number of reagents (7-12) which can affect the activity, and using different substrates (12-17) including ATP analogues (18,44).

Blum (16) showed a clear distinction in substrate behavior between the ATP class and the ITP class of substrates upon the enzymatic action of myosin. Based on his observations, Blum proposed the hypothesis that the amino group at the 6-position of the purine ring plays an important role in the interaction with the enzyme, causing a conformational change around the active site. This mechanism may bring about a rate-

retarding step during the hydrolysis of ATP. This hypothesis is further supported by Blum's recent work (19), by Gilmour's (20) and by Levy and Koshland's (21, 22) O^{18} exchange experiments which suggested the existence of an intermediate stage.

Based on our recent observation (23, 24, 45) on this problem, we have tried to re-examine the Blum-Gilmour theory of myosin nucleotide triphosphatase. In the discussion section a new model of active site and scheme of enzymatic reaction have been introduced. Several experiments have been done in order to prove the proposed theory, at least in part.

METHODS

Purification of myosin, AET treatment and method of phosphate determination for the ATPase activity have been carried out by the same way which was described in previous paper (23), *i.e.*, in most cases, activity was measured in a tetramethylammonium chloride (TMAC) solvent. Reaction was stopped by adding 20% perchloric acid and stannous chloride was used as a reducing reagent. In the pH range 6-7, histidine 0.05 *M* solution, around pH 8, Tris and pH above 9, alanine, have been employed as the buffers. The number of SH groups in myosin was determined by a PCMB spectrophotometric titration, *i.e.*, Boyer's method (25). Relative solubility was tested by mixing original myosin solution (1.4% 0.6 *M* KCl) to 10 times the volume of indicated ionic strength solvents and centrifuged at $30,000 \times g$ for 30 minutes. U.V. absorption at 280 $m\mu$ of the supernatant were compared with an 11-fold dilution of the original solution.

RESULTS

1. SH Groups in Myosin and their Reactivity

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Abbreviations used here are:

PCMB	<i>p</i> -chloromercuribenzoate
AET	S- β -aminoethylisothiuronium
PP	pyrophosphate
TMAC	tetramethylammonium chloride
U.V.	ultraviolet light
NTP	nucleotide triphosphate
TBS	2,4,6-trinitrobenzene-sulfonate

with PCMB—When we mixed the myosin solution and PCMB solution which contains less equivalent moles of PCMB than the moles of SH group in myosin, reaction between them completes within a very short time and optical density at $250\text{ m}\mu$ does not change due to the time. However, if equivalent or more amounts of PCMB than the SH groups in myosin were added, slow increase of U.V. absorption can be observed for long periods of time after completion of rapid reaction (Fig. 1). This phenomenon may be under-

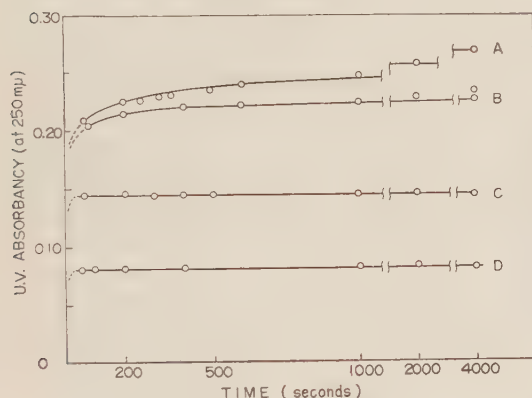


FIG. 1. Titration of SH groups in myosin A by PCMB. Time dependency of U.V. absorption ($250\text{ m}\mu$) at various concentration of PCMB. Myosin concentration 0.75 mg./ml.

PCMB concentration:

- A $1 \times 10^{-4} M$, about 1.5 times equivalent moles of SH groups in myosin.
- B $0.7 \times 10^{-4} M$ about equivalent moles of SH groups in myosin.
- C $0.4 \times 10^{-4} M$ about $2/3$ equivalent moles of SH groups in myosin.
- D $0.2 \times 10^{-4} M$ about $1/3$ equivalent moles of SH groups in myosin.

stood if we assume there are two kinds of SH groups in myosin. Number of moles of SH groups in 10^5 g. of myosin decrease to about a half of original value (23) after AET treatment and treated one also shows increase of U.V. absorption with excess PCMB.

On comparing the results of PCMB titration before and after AET treatment of myosin in the presence of pyrophosphate, we observed in both cases, approximately the

TABLE I

Number of Moles of SH Groups in Myosin before and after AET Treatment
Absence and presence of pyrophosphate. Concentration of myosin A 0.75% in 0.6 M KCl
pH 8 Pyrophosphate $2 \times 10^{-3} M$
MgCl₂ $5 \times 10^{-3} M$

	Moles SH per 10^5 g. myosin	Moles SH per 10^5 g. myosin pyrophosphate	difference
Original myosin	6.9	6.4	0.5
AET treated myosin	3.4	3.0	0.4

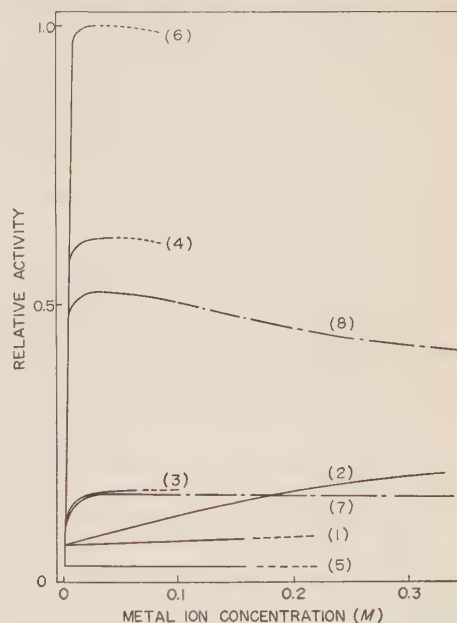


FIG. 2. Effect of metal ions on ATPase activity of myosin B. Conditions same as Table II.

(1) Activity in tetramethylammonium chloride (TMAC). No metal ion present. Abscissa indicates concentration of TMAC. Activity is very small at any ionic strength.

(2) Activity with KCl in TMAC. Total ionic strength 0.6 M . Abscissa shows concentration of KCl.

(3) Activity in 0.6 M TMAC with Mg^{++} .

(4) Activity in 0.6 M TMAC with Ca^{++} .

(5) Activity of AET-treated myosin, in TMAC. Conditions same as in (1).

(6) Activity of AET-treated myosin in 0.6 M TMAC with Ca^{++} .

(7) Activity of original myosin with $10^{-2} M$ Mg^{++} . Abscissa shows concentration of KCl. Total ionic strength 0.6 M .

(8) Activity of original myosin with $10^{-2} M$ Ca^{++} . Abscissa shows concentration of KCl. Dotted lines indicate approximate tendency of the activity.

same number of PCMB titratable groups had been lost presumably due to the pyrophosphate SH group interaction (Table I). The values in Table I vary depending on preparation of myosin and condition of AET treatment, however, reproducibilities on the same preparation are quite satisfactory. Though differences in PCMB titratable SH groups in the absence and presence of pyrophosphate are very small, they are obviously bigger than the experimental error which is less than 2.5%.

2. *ATPase Activity in Tetramethylammonium Chloride (TMAC)*—In this solvent, salt effects would be purely due to the ionic strength because interactions between the ions and myosin are negligibly small. In the absence of any metal ions, myosin has virtually no enzymatic activity. The effect of Ca^{++} is much more remarkable than in KCl and

Mg^{++} also shows an activating action. (Table II, a,b). As shown in Table IIc, in the case of AET treated myosin, the effects of metal ions are emphasized. The results of Table II can be illustrated in Fig. 2 as the relative activities under various conditions.

3. *Comparison of Solubility in Tetramethylammonium Chloride and in Potassium Chloride*—The relative solubility at low ionic strength in TMAC and KCl at pH 8, 6.8 are shown in Fig. 3.

4. *Inhibition of ATPase, ITPase of Myosin by ADP*—ADP inhibits ATPase activity of myosin slightly, and ITPase activity remarkably. Ca^{++} -activated ATPase activity is accelerated by AET-treatment but Ca^{++} -activated ITPase activity is decelerated by AET-treatment. In both cases, the inhibitory action of ADP was desensitized by the AET treatment (Table III). This fact may indicate

TABLE II

Effect of Metal Ions on ATPase Activity of Myosin

Sample 5 hour extract myosin B. ATP concentration $10^{-3} M$ pH 8.5 25°C . Activity expressed as $\mu\text{moles PO}_4^{---}/\text{g. sec.}$

(a) In $0.6 M$ tetramethylammonium chloride (TMAC)

(b) In $0.6 M$ potassium chloride.

(c) After AET treatment, in (TMAC).

Conc. of metal ion M	0	0.64×10^{-4}	1.6×10^{-4}	0.8×10^{-3}	1.5×10^{-3}	10^{-2}	2×10^{-2}
Activity with Ca^{++}	0.33		1.12	4.25	5.25	5.45	5.12
Activity with Mg^+	0.33	0.137	0.187	0.285	0.422	0.410	

Conc. of KCl	0	0.1	0.2	0.3	0.6		
Activity with K^+	0.31	1.20	1.36	1.56	1.82		

(a)

Conc. of metal ion M	0	10^{-5}	10^{-4}	10^{-3}	2.5×10^{-3}	5×10^{-3}	10^{-2}
Activity with Ca^{++}	1.81		2.08	3.01	3.45	3.80	3.80
Activity with Mg^{++}	1.81	1.03	0.19	0.11			

(b)

Conc. of metal ion M	0	0.64×10^{-4}	1.6×10^{-4}	0.8×10^{-3}	1.5×10^{-3}	10^{-2}	2×10^{-2}
Activity with Ca^{++}	0.108			10.64	12.38	14.50	16.98
Activity with Mg^{++}	0.108		0.417	0.620	0.700	0.790	0.952

(c)

that by the treatment, the interaction between ADP and myosin has been decreased.

DISCUSSION I

I. SH Groups in Myosin—Since myosin ATPase responds very sensitively to SH reagents and heavy metal ions which primarily bind to SH groups in myosin, it is almost certain that SH groups, with their neighboring configurations, are involved in its enzymatic activity. There is fair evidence for the existence of at least two kinds of SH groups—in the sense of reactivity with SH reagents, and in regard to their role in enzymatic action. During SH group titration by PCMB, we found two phases of reaction rate, *i.e.*, fast and slow reacting groups exist in myosin. Gellert and Gilmour* have studied this phenomenon kinetically in detail. According to their results, elimination of fast reacting groups does not decrease ATPase but rather increases it. This may mean that fast reacting SH groups are not involved in the hydrolytic process of ATPase. By treatment at high pH with AET (9), myosin always loses about one-half of its SH groups, and this is accompanied by tremendous activation of ATPase over the whole pH range with Ca^{++} (23). If we suppose AET reacts with only one kind of SH group, namely the fast reacting group with PCMB, this phenomenon can be easily understood. On the contrary, iodoacetoamide-treated myosin loses all activity when about half of its SH groups are tied up (12). This reagent seems to react rapidly with the SH groups which react slowly with PCMB, *i.e.*, active groups. We have demonstrated that in the presence of pyrophosphate, the number of PCMB-titratable groups decreased by about 6 per cent (23). As shown in Table I, in the AET treated myosin case, we observed that the number of reductions of PCMB-titratable groups in presence of pyrophosphate is the same in spite of the fact that AET treated myosin contains only a half of SH groups in original myosin. This may mean that

AET does not interact with the same groups with which PP interacts.

It is possible to conclude that there are two kinds of SH groups in myosin from both chemical and enzymatic observations. The unification of these observations brought about by supposing that SH groups which react slowly with PCMB, react rapidly with iodoacetoamide, bind with pyrophosphate and do not react with AET are those involved in the hydrolytic process as well as interaction between substrate and myosin molecules. The other kind of SH group (fast reacting with PCMB, reacting with AET, not interacting with pyrophosphate, and reacting slowly with iodoacetoamide) is that involved in the strong binding of the other (ring) end of the substrate—this binding actually being an inhibitory factor as far as ATPase rate is concerned. This idea is supported by the fact that K decreases after treatment with PCMB (19). The other kind of SH groups would participate in the hydrolysis of substrates as well as in binding. The two kinds of SH groups must be located near each other on the active site so that substrates can reach both simultaneously

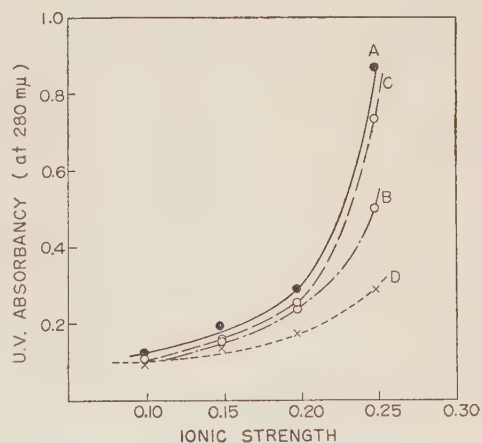


FIG. 3. Comparison of solubility of myosin in KCl and TMAC. Original concentration; 1.3% in 0.6 KCl. 11 times dilution, U.V. absorption at $280\text{m}\mu$ 1.00. Relative absorbance at $280\text{m}\mu$.

	in KCl		in TMAC
pH 8.0	A —●—		C —○—
pH 6.8	B —○—		D —×—

* Gellert, M., and Gilmour, D., Personal communication.

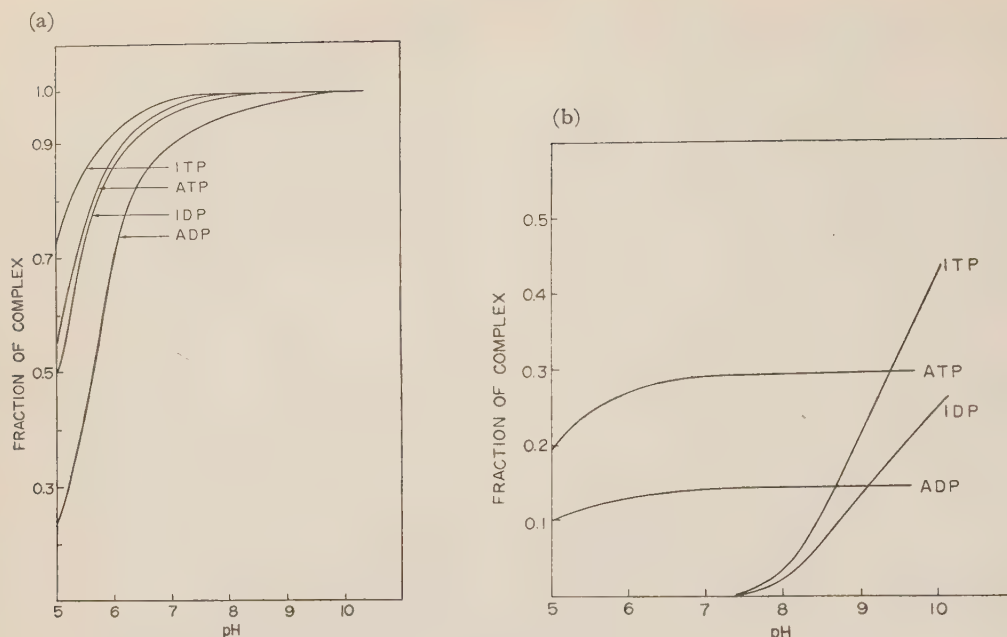


FIG. 4. Fraction of NTP-Mg⁺⁺ complex at various pH

(a) The fraction of overall complex. NTP, $10^{-3} M$ Mg⁺⁺ $10^{-2} M$

(b) The fraction of complex at the ring side. NTP, $10^{-4} M$ Mg⁺⁺ $10^{-3} M$

If we assume K_m , K_h as affinity constants between metals and nucleotides and reciprocals of ionization constants, the fraction of complex can be expressed as,

$$\frac{K_m [M]}{1 + K_m [M] + K_h [H]}$$

$[M]$, $[H]$, represents concentration of metal ion and hydrogen ion, respectively. Hotta, Brahm, Morales (45) gave $K_{m(ATP)} = 8 \times 10^3$, $K_{m(ITP)} = 1.2 \times 10^4$ for overall affinity constants and $K_{m(ATP)} = 0.42 \times 10^3$, $K_{m(ITP)} = 1.4 \times 10^3$ for ring side affinity constants. Using this value, we can calculate each fraction at various pH's.

without causing shape change of whole molecule.

II. Effect of Salts and Metal Ions—ATPase activity of myosin is greatly affected by varying the concentration of salt in the solvent. Two factors may contribute to this. One is ionic strength and the other is specific interaction between the ions and the protein. In potassium chloride solvent which is the most commonly used, there may be some interaction between K⁺ and protein resulting in some effect on enzymatic and other properties of myosin. Actual evidence which indicates that K⁺ can interact with protein and substrates has been shown (26, 27). Solubility of myosin B is different in an inert electrolyte (TMAC) and KCl solvent (Fig. 3).

It is very useful to use an inert electrolyte, such as tetramethylammonium chloride (TMAC) (23) as a solvent so that we can distinguish the two kinds of salt effect (ionic strength and specific interaction) without any complications (23). At any ionic strength of TMAC, it is found that myosin has only a very small activity in the absence of added metal ion. Ca⁺⁺ activates activity more remarkably than in KCl. Mg⁺⁺ is a weak activator and activation by K⁺ is clearly shown in Fig. 2. We may think that any kind of metal ions could be activators in this medium. In AET treated myosin B, activation with metal ions is much more emphasized than in non-treated myosin.

These results prove that the presence of

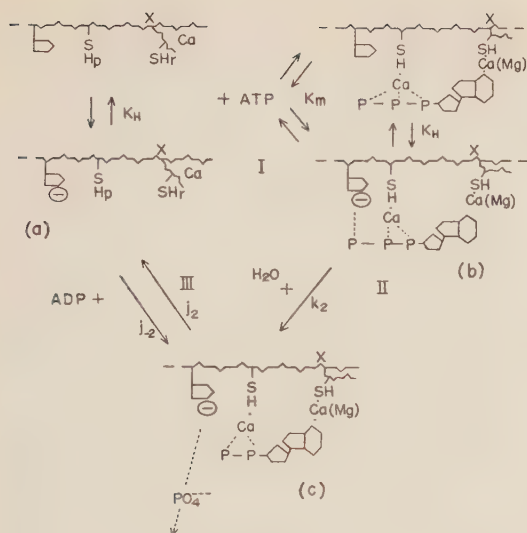


FIG. 5. Model of active site and mechanism of catalysis.

SHP phosphate interacting SH group

SHR ring interacting SH group

X unidentified amino acid at the connecting position of SHp and SHR.

ionized imidazole group.

unionized imidazole group.

metal ion is a requirement for the enzymatic action of myosin, presumably for the formation of enzyme substrate complex. Nihei and Tonomura (28) have examined the effects of several other metal ions and found a beautiful correlation between activity and ionic radii of metal ions. However, as pointed out by Blum* (19), heavy metals react with SH groups and possibly other groups, giving many complicated results. We cannot correlate their activation or inhibition effect simply with ionic radii without counting irreversible inactivation of enzyme.

From the foregoing observation, we may conclude that higher ionic strength gives higher activity and simple behavior by damping out the field around the site. Ca²⁺ might be essential as a co-factor for the

enzymatic activity of myosin by forming a bridge between enzyme and substrate at the hydrolytic site. Mg²⁺ helps to form enzyme substrate complex not at the hydrolytic site but at another position, presumably at the ring end. When Mg²⁺ interacts the hydrolytic site, however, the rate of hydrolysis is much slower than in the case of Ca²⁺.

III. Substrate Specificity—Myosin can hydrolyze several kinds of nucleotide triphosphate and tripolyphosphate. At the same time, these substances can be discriminated by myosin with a different rate of hydrolysis and Michaelis-Menten constants. Extensive work has been done by Blum (16, 19) in order to characterize the activity of myosin on different substrates. He conclusively showed that most of the difference comes from the difference in reactivity of ring NH₂ groups (6-position in purine rings and 4-position in pyrimidine rings) and ring OH groups (ditto). Tonomura (44), Moos (18) synthesized several ATP analogues and examined enzymatic action of myosin on these substances. According to their results, so far as enzymatic hydrolysis is concerned, ring or ribose base modified ATP analogues are not remarkably different from the original one. These modified at the phosphate side do not do anything to myosin nor are they hydrolyzed. From those facts, any substrates and substrate analogues which have triphosphate can be hydrolyzed by myosin regardless of what structure they might have at the other ends. Interaction at the ring side to the protein gives additional affinity causing some complication on the velocity of hydrolysis. It may accelerate by forming enzyme substrates complex or decelerate by preventing products from desorption.

We showed some evidence (45) that at neutral pH, interaction between the 6-amino group of the purine ring and Mg²⁺ would be much greater than that of the 6 hydroxy group in purine ring, just as observed in the myosin-metal-substrate system.

As shown in Table III, ATPase activity of myosin B was slightly inhibited by the presence of ADP, however, the same concen-

* Blum, J., Personal communication.

tration of ADP can inhibit ITPase strongly. This is probably due to the fact that strong interaction between myosin and ADP at the ring end prevents the approach of the ITP molecule to myosin. AET treatment reduces the interaction between SH groups and the adenine ring, so we can expect that specificity of substrate would be eliminated. Inhibitory action of ADP decreases in both ATPase and ITPase, especially in the latter case after treatment with AET (Table III). With AET

TABLE III

	ATP only	ATP+ADP	Ratio
Activity original	3.39	3.05	0.90
Activity AET treated	14.8	14.0	0.95

(a)

	pH	ITP only	ITP+ADP	Ratio
Activity original	6.3	16.42	3.80	0.23
„	7.2	19.50	6.60	0.31
„	9.0	23.90	6.73	0.28
Activity AET treated	6.3	1.20	0.62	0.52
„	7.2	1.66	1.26	0.76
„	9.0	2.35	1.44	0.67

(b)

Inhibition of ATPase, ITPase of Myosin B by ADP before and after AET treatment. In 0.6M TMAC, concentration of ATP, ITP, ADP, 10⁻³M. pH 6.3, 7.2, 9.0 25°C. Activity expressed by μM PO₄⁻⁻⁻/g. sec.

(a) ATPase

(b) ITPase

treated or PCMB treated myosin (20, 23) ATPase shows smooth pH dependency just like ITPase shows. Kinetic constants for treated myosin are about the same in magnitude for ATPase as they are with intact

myosin for ITPase (19). The Mg⁺⁺ effect is insensitized and Ca⁺⁺ effect emphasized, *i.e.*, if we eliminate ring end-enzyme interactions, most of the nucleotide specificity will disappear. Tonomura's group (44) and our observations (23), generally speaking, seem to support Blum's and Gilmour's (16, 19, 20*) ideas which emphasize the importance of ring and interaction to the enzyme.

IV. Interaction Between Substrates and Metal Ions—Complex formation between nucleotide phosphates and metal ions has been well known. Several papers have been published on quantitative measurements of affinity constants between several nucleotide phosphates with Mg⁺⁺ and Ca⁺⁺ (29-32, 45), but agreement among the authors is rather poor. As shown previously, metal ions are required for enzymatic activity of myosin. It is reasonable to assume that the phosphate end of the substrates interacts with the active site of the protein through metal ions during enzyme-substrate complex formation. We have shown (6) that Mg⁺⁺ and Ca⁺⁺ can bind not only to the phosphate side, but also with both purine and pyrimide rings of substrate molecules and that they do not bind with inosine, adenosine and cytidine bases. These observations strongly indicate that bound divalent cations to the phosphate side can interact to the ring, presumably forming curl structure of the molecule (33-35). It is interesting to point out that in both overall and ring affinity to Mg⁺⁺, ITP is superior to that of ATP (Fig. 4). However, because of the difference of ionization constants of NH₂ and OH groups in purine ring, metal complexes with the adenine ring are much stronger than with the inosine ring at the conventional pH where ATPase is usually measured. As shown in Fig. 4 (b), the fraction of Mg⁺⁺ ATP complex at the ring end is much greater than that at neutral pH of ITP.

Considering these facts, we may relate this ring end metal interaction difference between ITP and ATP to the actual nucleo-

* See the foot-note of page 223.

tides-protein systems and can understand the strong inhibition of ITPase of myosin by ADP (Table III).

V. Intermediate Myosin Phosphate Complex and the Rate of Hydrolysis—During the course of hydrolysis, the formation of intermediate enzyme-substrate complex has been suggested in the theory of Blum and Gilmour (16, 19, 20) in terms of "active complex" related to conformational change of the active site in myosin. Levy and Koshland (21, 22) demonstrated that O^{18} exchange of inorganic phosphate can be catalyzed by myosin in the intermediate stage of ATP hydrolysis and proposed that the dephosphorylation of ATP passes through the stage of a phosphorylated myosin. However, Gergerly and Maruyama (36) showed that inorganic phosphate not developing from ATP can bind to the enzyme during ATPase activity. Our proposed scheme which is shown below might explain these results.



After ATP is hydrolyzed, Pi removed from enzyme faster than the other product, ADP. Velocity of desorption of ADP would be the important factor influencing overall reaction rate.

Important information was obtained by us (24) to determine which is the rate-limiting process by using D_2O . ATPase and ITPase activity are considerably reduced in D_2O presumably due to a slow hydrolytic reaction with D_2O . However, we ascertained that at pH around 7.2 where usually ATPase activity is minimal, the rate of hydrolysis of ATP in D_2O is exactly the same as in H_2O . Therefore, we can assume except around pH 7.2 for ATP, rate-limiting step is the hydrolytic process, II, in our scheme. It is clear that around neutral pH for ATP, process III becomes rate-limiting because attack by H_2O and D_2O does not make any difference to the overall reaction rate. Myosin ADP complex is stabilized around pH 7.2 by some mechanism presumably due to the interaction between myosin and the ring end of ADP.

The IDP molecule, on the other hand, does not have such a strong affinity. AET treated myosin does not have such a minimum ATPase activity at neutral pH both in H_2O and D_2O . Elimination of ring side interaction accelerates overall reaction by increasing the velocity of desorption of products at neutral pH (process III).

VI. Responsible Groups in the Active Site—

It is almost certain that SH groups are involved, together with their neighbors. Besides SH groups, Bárány and Bárány (12) and Tonomura *et al.* (11, 46) showed elimination of NH_2 groups causes inhibition of ATPase activity. 2,4,6-Trinitrobenzenesulfonate (TBS), the reagent which was used by Tonomura's group, attacks ϵ -amino groups of lysine giving considerable change in myosin ATPase activity. The role of NH_2 groups is not clear, though it is natural to assume that there may be no direct interaction between substrates and ϵ -amino groups because its effect greatly depends upon the ionic strength of the media (11, 46), however, lysine groups must be located near the active groups, for instance, SH groups, and must interfere with the substrate enzyme interaction.

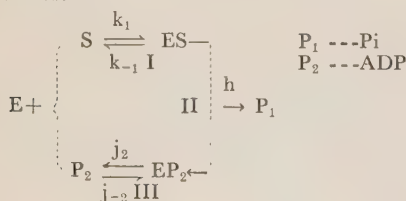
Morales and Hotta (23) introduced the imidazole groups as a possible candidate. With AET treated myosin B, in high ionic strength of TMAC, ATPase-pH curve is identical to the ITPase curve indicating single group ionization. From the pK value of this group which is 6.8 at 25°C and ionization heat of several kilocal., we may consider that this is a reflection of histidine ionization. Noda, Nihei and Morales (37) observed the same shape of pH-activity relationship with ATP-creatine transphosphorylase activity. However, these facts may not mean that myosin or creatine kinase action is related to the mechanism of chymotrypsin, since diisopropylfluorophosphate (DFP) which is a strong inhibitor for chymotrypsin, does not give any effect on myosin ATPase (23).

Another important indication that a histidine group is involved in muscle activity has been brought forth by Edman (38). He measured inhibition of contractility in

the presence of Zn^{++} at various pH and obtained a titration-like curve with pK about 6.8. This implies an excellent correlation between the number of intact imidazole groups and the contraction of the muscle model. If we interpret contractility in terms of myosin ATPase, our observations of AET treated myosin B and Edman's on muscle model might stem from the same group. This hypothesis suggests that the active site for ATPase of myosin is involved in the contraction activity of muscle.

DISCUSSION II AND CONCLUSION

The nature of the enzyme-substrate complex formation at the molecular level is unknown; however, among other factors, we can assume safely that chelation of substrate to the tightly bound metals on the protein and electrostatic repulsion or attraction is one of the main causes of interaction between them. Absorption of substrate or desorption of products may be regulated by competition and balance of those factors. If we assume as a first approximation that the metal substrate interaction is the same as protein substrate interaction, we can figure out to some extent the nature of protein and substrate interaction. Metal binding to the myosin also increases with pH (38, 39). This factor tends to increase complex formation. On the other hand, the charge on the substrate and products, which presumably gives rise to repulsive forces, also depends on pH. As we see very easily, one of the products, PO_4^{---} which has poor affinity for the metal ($K_m=76$ (29)) and relatively high charge, is easily taken out of the enzymatic site after hydrolysis. In the ATPase case, we may consider that ADP which is one of the products is a competitive inhibitor. We can express this situation as follows.



At the steady state, velocity of hydrolysis is:

$$V = \frac{h \frac{k_1 [S]}{k_{-1} + h} E_0}{1 + \left(1 + \frac{h}{j_2} \right) \frac{k_1 [S]}{k_{-1} + h} + j_{-2} [P_2]}$$

Maximum velocity can be reached at $[S] \rightarrow \infty$ and $[P_2] \rightarrow 0$.

Then, $V_{\max} = hE_0/(1 + h/j_2)$.

Two cases can now be distinguished. (a) If $h \gg j_2$, then $V_{\max} = j_2 E_0$, that is $V_{\max}$ will no longer be affected by changes in h . (b) If $h \ll j_2$, then $V_{\max} = hE_0$, that is, any changes in h will be reflected directly in $V_{\max}$. We believe that substitution of D_2O for H_2O is a method of reducing h , and that treating the enzyme with AET (or comparable treatment with PCMB, DNP, *etc.*) is a method for increasing j_2 . For example, for ATPase around neutral pH, we think $h > j_2$, so that case (a) is approximated and substitution of D_2O has no effect on $V_{\max}$. However, if the enzyme has been pre-treated with AET, j_2 has been so increased that case (b) is approximated, and now the D_2O substitution does reduce $V_{\max}$. At non-neutral pH's for ATPase, and at all pH's for ITPase, we feel that case (b) is always approximated.

As mentioned previously, the active site contains two SH-bearing residues—a "phosphorylating SH", SHp, and an SH which interacts with the ring, SHr—and a histidine residue, H. These residues must be close to one another, and in a particular configuration. According to Snellman (40), when all Ca^{++} is removed from myosin it is possible to extract a peptide chain containing SH and lysine residues. Snellman determined partial amino acid sequences, and considered, with several other evidence, that this chain was at least part of the active site. Since Snellman's chain is fairly easily removed from myosin it is probably rather loosely connected to the main structural chain by Ca^{++} . We may speculate that the residue arrangement is that of Fig. 5(a). The configuration of the portion of the chain bearing SHr is thus easily adjusted by metal binding or by varying ionic strength (and thereby perturbing electric fields). Picture of mechanism of reac-

tion can be drawn as Fig. 5. When a substrate molecule approaches this site it interacts with it through the metal ions at SHp and also at SHr, depending on pH and on the nature of the substrate ring (Fig. 5(b)). In the next step the imidazole residue comes into contact with the terminal phosphate group and in some way catalyzes hydrolysis; according to the evidence cited above only the ionized form of the imidazole residue functions in this process (Fig. 5(c)). In the absence of complicating interactions this is followed by desorption of products (P_2). However, around pH 7, the ADP-myosin complex may be stabilized by interactions between ADP and both SHp and SHr; if so, this situation prevents immediate replacement by ATP, and thus retards the overall reaction rate. If we assume that the (nucleotide) ring-metal interaction in the absence of enzyme behaves like the (nucleotide) ring-metal-enzyme interaction, then, up to pH 9 at least, the stabilization of the IDP-myosin complex would be very weak. In the absence of these complicating stabilizations (*e.g.*, in ITPase, or in AET treated ATPase) we expect the velocity of hydrolysis to change with pH in about the same way that the concentration of ionized imidazole residues changes with pH (23). In our model the distance between SHp and SHr is adaptable to the dimensions of various substrates. Several analogues differing slightly from ATP in dimensions can thus be hydrolyzed by the same mechanism as ATP. However, product stabilization cannot always be expected. Analogues in which the polyphosphate chain has been modified are treated very differently from ATP; presumably the dimensional requirements in the H-SHp region are very strict.

Myosin contains many (63 moles, if the myosin molecular weight is assumed to be 4.2×10^5) histidine residues and many (34) cysteine residues (4). Obviously all of these are not members of active sites; indeed the number of active sites in myosin has been reported to be of the order of one or two. We think that when these three responsible groups take a configuration such as that

described above, an active site would be formed. Adopting a myosin model, such as that suggested by Kielly and Harrington (42), we may calculate the probability that the three groups simultaneously be in a region having the dimension of the substrate molecules. Kielly and Harrington suggested a myosin molecule consisting of three identical chains wound helically about each other. We may assume on the way how to arrange the three chains together, *i.e.*, phase of each chain relative to the other two are at random when random relative sliding is considered. Based on this assumption, we get roughly unity as the number of active sites per molecule*. Obviously, this is not positive support of the proposed model and agreement with experiments might be purely coincidental. However, we can get an impression that active site essentially means such a sequence of responsible groups.

The relation of myosin ATPase to muscle contraction has not been examined in this paper. It is, however, implied in all models hereto proposed, for instance in Podolsky's (43), wherein both the steady state isometric tension and the velocity of isotonic shortening would depend in part on the rate at which ATP molecules can be hydrolyzed by myosin off the thin contractile filaments. Edman's

* In the Kielly-Harrington myosin model (42), we suppose that each chain contains 1,200 amino acid residues, 12 SH groups, 22 histidine groups. Taking into account that any residue on a helically wound chain has, say, 8 neighbors which are members of the 'same' chain the probability that a histidine and an SH group be neighbors will be

$$\frac{22 \times 12}{(1200)^2} \times 8 = 1.47 \times 10^{-3}$$

There are three such chains considered identical. Therefore this number would be 4.41×10^{-3} in one molecule. Thus, opportunity that another SH group come near this position (we assume also 8) would be written as follows under the assumption that this SH group could be on one of any three chains:

$$4.41 \times 10^{-3} \times \frac{12}{1200} \times 8 \times 3 \times 1200 = 1.2$$

This means about one active site can be expected per molecule.

results (38) are also a reflection of this relation, as are the weakness of ITP contractions, the relaxing effect of SH reagents, Mg^{++} acceleration and Ca^{++} inhibition.

SUMMARY

1. Two kinds of SH groups were defined chemically and enzymatically, *i. e.*, difference in reactivity with the SH reagents and interaction with substrates.

2. Role of divalent cations, especially as the chelaters of substrates have been discussed showing the evidence that the existence of interaction between the ring and groups and Mg^{++} or Ca^{++} .

3. Rate-retarding steps of myosin NTPase among the consecutive enzymatic process are suggested. For ATP class, at neutral pH, rate-retarding step is desorption of ADP. For the other pH, AET treated myosin for ATP, and ITPase case, that is hydrolytic process.

4. Histidine group introduced as a possible group for NTPase of myosin from the pH dependency of enzymatic action and contractility of muscle model.

5. Model for active site is proposed as a combination of two kinds of SH groups and histidine group. One of the SH groups and the histidine group form the hydrolytic site and one SH group makes the substrate binding site.

6. Relation between ATPase and contraction mechanism of the muscle model were discussed briefly.

I would like to express my sincere thanks particularly to Prof. M. F. Morales for valuable suggestions and discussions. I also wish to thank Dr. Watanabe for helpful discussions, and Drs. Blum and Tonomura for communicating to me their unpublished data.

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Stereospecific Reduction of Capillin, an Antifungal Substance, by Mold Mycelia

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Polyene antibiotics have been reported to be active against yeasts and fungi (1), while synthetic α , β - ethylenic ketones such as acrylophenone and dibenzoyl ethylene have been known to show relatively broad antimicrobial spectra (2).

An antibiotic, capillin, isolated in the author's laboratory from the essential oil of *Artemisia capillaris* Thunb. was identified to be 1-phenyl-1-oxo-hexa-2,4-diyne (3) and was found to be highly active against various yeasts and fungi but not against bacteria (4). Thus this benzoyl polyne antibiotic was revealed to be similar to polyene antibiotics in its antimicrobial spectrum.

It may be emphasized that capillin has a carbonyl group conjugated with diacetylenic linkage in its molecule. Some informations available at present indicate that such carbonyl group is different in chemical reactivity from other carbonyl groups. This fact suggests that the carbonyl group in capillin molecule may in part be responsible for the exhibition of the antimicrobial activity. In fact, it was already observed (5) that carbonyl group in some capillin analogues was essential for their antimicrobial activity.

In the present investigation, the author found that capillin was reduced to the corresponding hydroxyl compound by fungal mycelia. The capacity of various microorganisms to reduce capillin was also examined with special reference to its antibiotic spectrum.

MATERIALS AND METHODS

Capillin and Capillinol—Samples used are those synthesized in this laboratory (3). Prior to experiments, capillin and capillinol (corresponding hydroxyl

form of capillin) were recrystallized from *n*-hexane and a mixture of *n*-hexane and chloroform, respectively.

Test Organisms and Cultural Conditions—Test organisms and cultural conditions for obtaining cell suspensions, unless otherwise specified, are listed in Table II.

Spectrophotometric Determination of Amount of Capillin—In experiments demonstrating the capacity of reduction of capillin, spectrophotometric method was employed. Cell suspension was extracted with *n*-hexane and the amount of capillin in hexane layer was determined by the estimation of the absorbancy at 276.5 $m\mu$, which is characteristic absorption maximum of this antibiotic.

EXPERIMENTALS AND RESULTS

Reduction of Capillin by Mold Mycelia—When capillin was added to mycelial suspension of

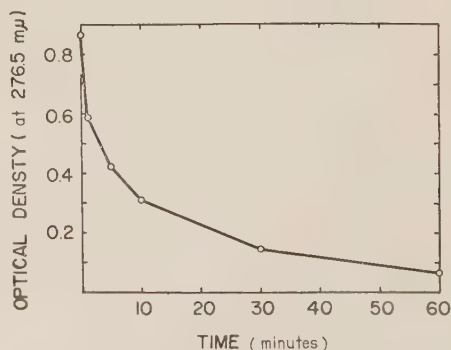


FIG. 1. Time course of loss of capillin in mycelial suspension of *Penicillium italicum*.

Washed mycelia (430 mg. in wet weight) was suspended in 87 ml. of medium B, pH 6.0 (see foot note of Table II) supplemented with 4 $\mu\text{g.}/\text{ml.}$ of capillin, and incubated at 26°C. At intervals an aliquot was sampled and filtered immediately, then 8 ml. of this filtrate was shaken with 5 ml. of *n*-hexane. After dehydration with Na_2SO_4 , absorbancy of hexane layer at 276.5 $m\mu$ was determined.

a mold being susceptible to this antibiotic, definite decrease in the amount of capillin in the hexane extract of the filtrate was observed (Fig. 1). This suggested the occurrence of either absorption or decomposition of capillin by the mold mycelia.

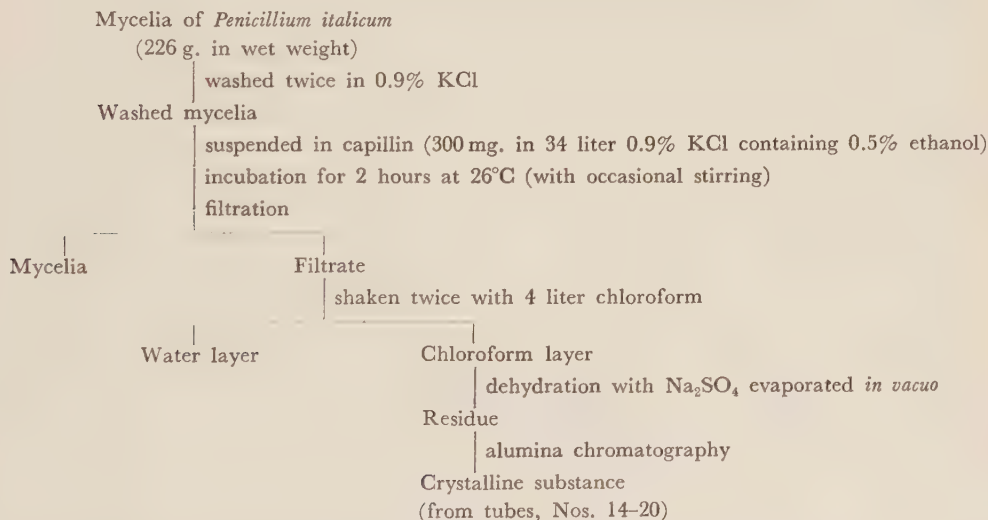
Examination of the ultraviolet spectrum of the ether extract of the filtrate revealed the appearance of new two absorption maxima (at $241\text{ m}\mu$ and $255\text{ m}\mu$ in ethanol*), although the intensity of the absorption was very weak. Moreover, as a result of infrared spectrophotometry of the ethylacetate extract of the filtrate, the absorption band at $2,270\text{ cm}^{-1}$ for $\text{C}\equiv\text{C}$ stretching vibration (6) was clearly demonstrated. Thus, it was ascertained that acetylenic linkages in the solute

to be susceptible to capillin, were used as test organisms. The procedure for isolation of the compound is shown in Scheme I.

Penicillium italicum was shaken in medium B (see foot note of Table II) for 2 days at 26°C . Freshly harvested mycelia (226 g. of wet weight) were washed, added into capillin (300 mg.) solution and kept for 2 hours. Under these conditions, almost all part of capillin added was transformed to other acetylenic compound. The filtrate of this suspension was then extracted with chloroform. After the evaporation of solvent, the residue was then subjected to chromatography with neutral alumina and solvent systems shown in Fig. 2. As shown in this figure, unknown yellow substance (liquid,

SCHEME I

Procedure for Isolation of a Crystalline Substance



were still retained. From these observations, it was considered to be highly probable that capillin might be transformed into some other acetylenic compound, but not absorbed onto mold mycelia. Therefore, a compound derived from capillin was isolated from the filtrate and subjected to chemical and physical analyses. *Penicillium italicum* and *Piricularia oryzae*, both of which were known

probably of mycelial origin) was mostly eluted out with *n*-hexane*, and the addition of chloroform (1 part to 4 parts of *n*-hexane) resulted in the instant elution of the remaining unknown yellow liquid and another

* Main absorption maximum of capillin: $276.5\text{ m}\mu$ in *n*-hexane; $280\text{ m}\mu$ in ethanol.

* From the previous observation (7), it was ascertained that capillin can be eluted by *n*-hexane easily, when neutral alumina of grade III (8) was used. Thus unattacked capillin, probably of only minute amount, might be eluted out with *n*-hexane concomitant with a yellow substance.

unknown substance was then eluted. The latter was easily crystallizable after the evaporation *in vacuo* of the solvent. Thus 141 mg. of colorless needles were obtained.

From the results of elementary analysis of this crystalline substance*, it was suspected

Found: C, 84.73; H, 5.99 (per cent)

Calculated for capillinol ($C_{12}H_{10}O$): C, 84.68; H, 5.92 (per cent)

Further examination of ultraviolet absorption spectrum (Fig. 3) and infrared absorption spectrum (Fig. 4) of this crystalline

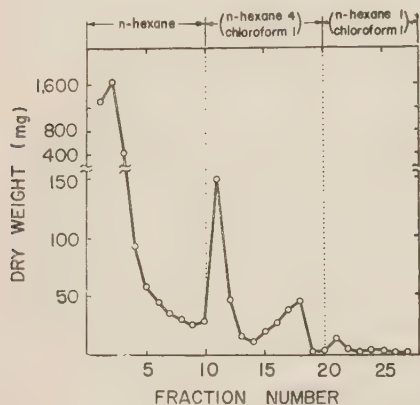


FIG. 2. Column chromatography of reaction product of capillin with mycelia of *Penicillium italicum*.

Column: neutral Brockman's alumina (grade III (8)), 10 cm. in height, and 2.5 cm. in diameter.

Solvent system: *n*-hexane, and mixtures of *n*-hexane and chloroform (4:1, 1:1).

Volume of each fraction: 30 ml.

Solvent of each fraction was evaporated *in vacuo* and dry weight of residue estimated.

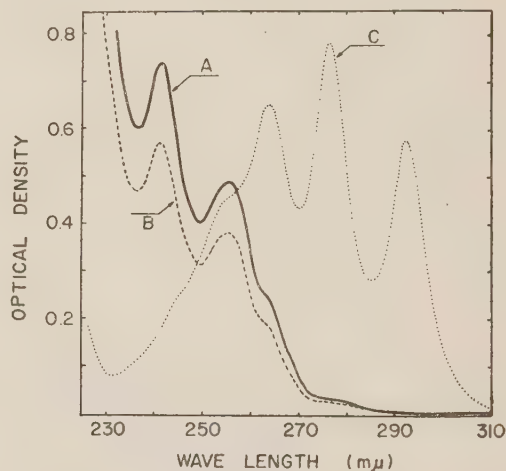


FIG. 3. Ultraviolet absorption spectra.

A: crystalline substance isolated as shown in Scheme I; sample recrystallized from mixture of *n*-hexane and chloroform, was used (15.6 mg./100 ml. in ethanol).

B: authentic capillinol (12.0 mg./100 ml. in ethanol).

C: capillin (0.57 mg./100 ml. in *n*-hexane).

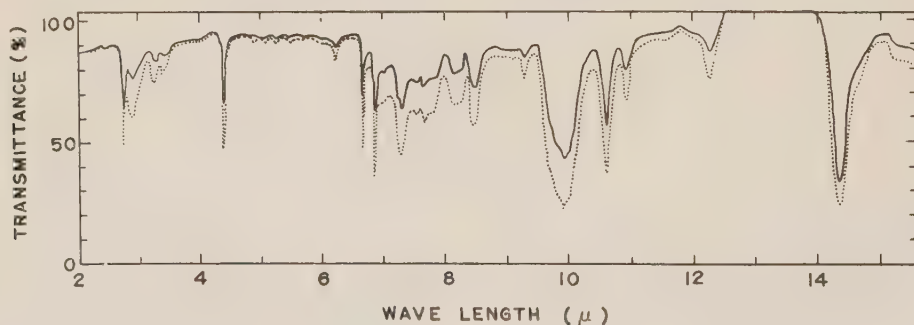


FIG. 4. Infrared absorption spectra.

Solid line: crystalline substance. Sample is the same one as used in Fig. 3 (in chloroform).

Broken line: authentic capillinol (in chloroform).

to be capillinol, 1-phenyl-1-hydroxy-hexa-2,4-diyne, as shown in the following data:

* A sample recrystallized from a mixture of *n*-hexane and chloroform, was used.

substance confirmed identity with authentic capillinol (synthetic).

It should, however, be mentioned that the melting point of capillinol obtained as described above was 104.5°C, while that of

TABLE I

Physical Constants of Crystalline Substance and
Authentic Capillinol (Synthetic)

Substance	Melting point	Optical activity
Crystalline substance ¹⁾	104.5°	$[\alpha]_D^{25} +12.1$ (c=3.15 in ethanol)
Authentic capillinol	86°	$[\alpha]_D^{26} 0$ (c=13.0 in ethanol)

1) Sample is the same one as used in Fig. 3.

Similar experiment was also carried out using *Piricularia oryzae* as a test organism. Thus 665 g. in wet weight of mycelia* of this organism was likewise reacted upon 150 mg. of capillin and 90 mg. of colorless needles were obtained by the isolation scheme as shown in Scheme I. All of the chemical and physical characteristics of this crystal were identical with capillinol produced by *Penicillium italicum*.

From these experiments one may conclude that mold mycelia can reduce capillin into an optically active isomer of capillinol as

TABLE II

Loss of Capillin in Cell Suspension of Various Microorganisms
and Antibiotic Spectrum of Capillin

Initial concentration of capillin was 5 μ g./ml.

Organism	Cultural condition ¹⁾			Amount of capillin disappeared (μ g./mg. of organism in dry weight/2 hours)	Minimal concentration required for complete inhibition (μ g./ml.)
	Medium ²⁾	Temperature	Duration (day)		
<i>Micrococcus lysodeikticus</i> IAM B-61-2	Broth agar	35°	1	0.7	50
<i>Streptococcus cremoris</i> IAM L-17	A	"	"	0.0	>100
<i>Sarcina lutea</i> PCI 1001	Broth agar	"	"	0.6	50
<i>Bacillus subtilis</i> ACTU B-5-6	"	"	"	1.1	100
<i>Lactobacillus casei</i> ϵ	A	"	"	0.1	100
<i>Escherichia coli</i> NIHJ	Broth agar	"	"	0.8	>100
<i>Saccharomyces cerevisiae</i> ATCC 9763	B	30°	"	3.1	2.5
<i>Saccharomyces carlsbergensis</i> ATCC 4228	"	"	"	5.6	5
<i>Zygosaccharomyces sauls</i> Z-3 ³⁾	"	"	"	4.8	5
<i>Penicillium italicum</i> IAM F-15-2	C	26°	2	5.3	2
<i>Aspergillus oryzae</i> A-8 ³⁾	"	"	"	3.5	5
<i>Glomerella lagenaria</i> G-8 ³⁾	"	"	"	5.5	10
<i>Alternaria kikuchiana</i> A-1 ³⁾	"	"	"	11.3	10
<i>Cochliobolus miyabeanus</i> C-36 ³⁾	"	"	"	>9.0	2

1) Yeasts and fungi were cultured by shaking.

2) Compositions of media were as follows (per liter)

A: 10 g. glucose, 10 g. sodium acetate, 10 g. yeast extract, 5 g. peptone, 250 mg. K_2HPO_4 , 100 mg. $MgSO_4 \cdot 7H_2O$, and trace amount of $FeSO_4$, $ZnSO_4$ and $MnSO_4$ (pH 7.0).

B: 20 g. glucose, 10 g. peptone, and 2 g. yeast extract (pH 5.4).

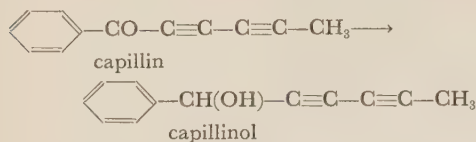
C: 20 g. sucrose and a potato extract obtained from 200 g. potato by boiling for 1 hour.

3) Stock culture in this laboratory.

synthetic capillinol was 86°C. Important is the fact that the capillinol obtained by the biological reduction shows definite optical activity, while synthetic one does not as listed in Table I.

* Obtained from a shaking culture at 26°C for 6 days in a medium, the composition of which was as follows: (per liter) 20 g. glucose, 5 g. peptone, 1 g. K_2HPO_4 , 0.5 g. $MgSO_4 \cdot 7H_2O$, 0.5 g. KCl, 10 mg. thiamine, 10 mg. biotin and trace of $FeSO_4$, $ZnSO_4$, and $MnSO_4$.

shown in the following formulae:



Transformation of Capillin by Various Microorganisms—From the results obtained by the preceding experiments, a question arises whether or not the capacity of a microorganism to reduce capillin is related to its susceptibility to this antibiotic. On examining this point, the degree of loss of capillin after the treatment with microbial cells of various species was determined spectrophotometrically, assuming that the loss of capillin may be caused by the reduction to capillinol.

Washed cells of various microorganisms were suspended in saline solution containing 5 $\mu\text{g.}/\text{ml.}$ of capillin to the cell density of 1 mg. dry weight of cells per ml. in bacteria or of 0.3–0.5 mg. dry weight of cells per ml. in yeasts and fungi. After 2 hours of incubation at 26°C, an aliquot of the suspension was taken and subjected to the extraction of capillin with *n*-hexane. The absorbancy at 276.5 $m\mu$ of hexane layer separated was estimated and the loss of capillin from the reaction mixture was determined*.

As shown in Table II, it was clearly demonstrated that bacteria which are insensitive to capillin, transform this antibiotic only slightly as compared with yeasts and fungi, which are highly susceptible to it. Thus the susceptibility of organisms to capillin seems to have a correlation to their capacity of its reduction to capillinol.

DISCUSSION

Present investigation revealed that microorganisms susceptible to capillin have a capacity to reduce this antibiotic at a carbonyl group to capillinol, a corresponding

* As shown in Fig. 3, molar absorption coefficient, ϵ , of capillin at 276.5 $m\mu$ is much higher than that of capillinol. Thus the decrease of absorbancy at this wave length represents that of capillin content, directly.

secondary alcoholic derivative of capillin. It was already found that capillinol was inactive against microorganisms, such as those susceptible to capillin. This fact seems to be paradoxical to explain the antibiotic activity of capillin. There is, however, such similar phenomenon that malachite green, a triphenylmethane dye active against bacteria, was found to be reduced by susceptible bacteria to leuco-malachite green, which was inactive against these bacteria (Yanagita, private communication). In such cases there may at least be two possibilities of mode of action: (a) the reduced compound itself is highly toxic against microbial cells when it is formed in a cell, and non-toxic when it is given from the outside of the cell and (b) the reducing reaction of the antimicrobial agent produces some unfavorable environmental conditions, such as, exhaustion of some important cofactors, change in rH, *etc.* Present experiment, however, seems to be still premature to answer these questions.

When an unsaturated linkage is introduced to a chemical structure adjacent to a carbonyl group, change in reactivity of the carbonyl group occurs, as being the case in α, β -ethylenic ketone. In the case of capillin, a carbonyl group is conjugated with diacetylenic linkage. Therefore, the carbonyl group in capillin molecule may also be different in reactivity from the carbonyl group in α, β -ethylenic ketone. This was well illustrated by the infrared absorption spectrum (3). As explained by Bellamy (9), the conjugation of a carbonyl group with ethylenic linkage results in a lowering of its absorption frequency. Capillin showed a band at 1,647 cm^{-1} for carbonyl stretching vibration. This value, indeed, is far less than those of α, β -ethylenic carbonyl groups (1,685–1,665 cm^{-1}). These facts suggest that such special character of carbonyl group in capillin molecule is highly responsible for the exhibition of its antibiotic activity.

As is well known, the acetylenic linkage is highly susceptible to chemical reduction. It was, however, found in the present investi-

gation that the carbonyl group in capillin molecule was specifically reduced by mold mycelia leaving acetylenic linkages unreacted. It is, therefore, probable to consider that such specific reduction may be also attributable to the special reactivity of the carbonyl group in the capillin molecule.

The most important finding in this investigation is the fact that mold mycelia reduce the carbonyl group of capillin stereospecifically. Recently, Prelog (10) reported that optically active secondary alcohols were produced from *ca* 70 kinds of keto-compounds through biological reduction by *Curvularia falcata* and these alcohols showed an uniform absolute configuration.

Such a stereospecific reduction of ketones in general may afford an important information on biological reduction.

SUMMARY

When capillin, a polyene antifungal substance, was incubated with freshly harvested mycelium of *Penicillium italicum*, its carbonyl group was found to be specifically reduced with resultant formation of a corresponding polyene alcohol, capillinol. Such a reduction was also observed by using *Piricularia oryzae* as a test organism. Important is the fact that the reduction occurred stereospecifically producing one of stereoisomers of capillinol.

In relation to the present finding, further investigations were carried out to examine the capacity of various microorganisms to reduce capillin. Remarkable reduction of capillin was observed in yeasts and fungi, whereas practically no reduction in bacteria

so far tested. Thus the reduction of capillin was considered to be correlated with the antibiotic activity of this antibiotic.

The author wishes to express his deep gratitude to Prof. T. Yanagita of Chiba University and Prof. A. Takamiya of University of Tokyo for their kind guidance and enthusiastic encouragement throughout this work. Thanks are also due to Prof. H. Umezawa of University of Tokyo for his valuable advice in the preparation of this paper, and to Mr. M. Matsui, director, and Dr. Y. Miura and Dr. I. Iwai of this Laboratory for their interest and encouragement. The author is indebted to Messrs. T. Onoe, O. Amakasu, and H. Higuchi, and to Misses C. Furukawa and H. Ohtsuka of this Laboratory, for elemental analysis and infrared spectrophotometry.

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Calcium Binding Activity of Vesicular Relaxing Factor

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Ebashi and Lipmann (1) have demonstrated that a purified preparation of the relaxing factor of skeletal muscle, shown by electron microscope to be a vesicular fraction**, probably the endoplasmic reticulum, is able to strongly bind calcium, and furthermore that this binding of calcium by the fraction depends on the presence of ATP***.

We have supposed that the calcium binding represents the physiological action, or the mechanism of the relaxing factor (2). The results demonstrated in the present paper support this concept, and suggest that the calcium ion is the main controlling factor in muscular contraction.

MATERIALS AND METHODS

Preparation of Actomyosin—Minced rabbit skeletal muscle was mixed with three volumes of 0.05 M KCl, homogenized for 1 minute with the Waring Blender and centrifuged for 10 minutes at $7,000 \times g$; to the residue were added four volumes of 0.6 M KCl containing 0.01 M NaHCO_3 . After 24 hours the extracted actomyosin was centrifuged at $20,000 \times g$ for two hours

and the residue was discarded. The supernatant was then slowly diluted by adding two volumes of cold water with gentle stirring to precipitate the actomyosin, and centrifuged at $7,000 \times g$ for 10 minutes. The residue was dissolved in sufficient M KCl to bring the final concentration of KCl to 0.6 M. It was then centrifuged at $20,000 \times g$ for 1 hour and the residue was discarded. To the supernatant, 0.15 M KCl was added while gently stirring to bring the final concentration to 0.25 M. The resulting solution was centrifuged for 30 minutes at $4,000 \times g$ and the centrifuged actomyosin was again dissolved in sufficient M KCl to bring the final concentration to 0.6 M. An equal volume of glycerol was added; this solution was stored in the deep-freezer at -18°C . This sample is called as "original actomyosin sample" in the present paper. All the procedures mentioned above were done below 4°C .

Preparation of Vesicular Relaxing Factor from Muscle—Prepared as described in the previous report (1). This was essentially the same as "twice-precipitated muscle extract" or P₂-M. E. in the earlier paper (3).

Superprecipitation of Actomyosin*—Superprecipitation was measured by following the increase in turbidity accompanying this phenomenon. The optical density at $660 \text{ m}\mu$ was determined mostly using Zeiss spectrophotometer. In cases of simultaneous determination of ATPase activity of actomyosin during its superprecipitation, Coleman's spectrophotometer, Model 6

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** Since the first isolation of the essential relaxing factor by Kumagai, Ebashi and Takeda (22), various names have been given to the factor, e. g., granular, microsomal and so on. In view of its microscopic structure, however, we preferred the name "vesicular relaxing factor" in this communication.

*** The following abbreviations were used in this paper: ATP, adenosine triphosphate; EDTA, ethylenediaminetetraacetic acid; EEDTA, ethyletherdiaminetetraacetic acid; GEDTA, glycoetherdiaminetetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; EDTA-OH, hydroxyethylethylenediaminetetraacetic acid; DTTTA, dimethyl-triethylenetetraminetetraacetic acid, and Tris, tris-(hydroxymethyl)-amino-methane.

* The term superprecipitation is defined in many ways by different investigators. We would accept the description, or definition, of Szent-Györgyi (4) which appeared in his earlier publication (1947)—"abundantly hydrated actomyosin molecules become a shrunken and dehydrated particle". This might indicate that the degree of superprecipitation would well be reflected in that intensity of the turbidity. Indeed, as shown in the results of the present paper, the increase of turbidity surely corresponds to the contraction, whereas the decrease corresponds to the relaxation. Furthermore, the results obtained with this method are more quantitative and reproducible than those with the experiments on glycerinated muscle fibers (Fig. 2).

was used.

To get a consistent result with this method, the actomyosin must be sufficiently finely flocculated prior to the addition of ATP to enable it to remain suspended without settling out until the turbidity reaches its maximum. Otherwise, spurious turbidity values, less than actual ones, would be read.

Determination of Calcium Binding Activities of Chelating Derivatives—Addition of chelating agents to the suspension of insoluble calcium oxalate results in the solubilization of certain amount of calcium corresponding to the calcium binding activity of each chelating compound. This phenomenon was applied to the determination of the actual calcium binding activity of chelating agents under the conditions of the relaxation experiments with muscle fibers described in the previous paper (5). Test solution consists of: 1 mM CaCl_2 containing $2.2\mu\text{C}$ of Ca^{45} per mmole Ca, 5 mM oxalate, 10 mM MgCl_2 , 0.14 M KCl, 0.01 M Tris-maleate-buffer (pH 6.8) and 1 mM chelating agent to be tested. After standing for 30 minutes at about 4°C , the solution was centrifuged and the supernatant was analyzed for its radioactivity of Ca^{45} . Calculation of apparent stability constants between calcium and chelating compounds were made under the assumption that the radioactivity above the blank value was due to the binding of calcium by the chelating compounds.

Determination of Calcium Binding of Vesicular Fraction—Procedures were the same as described in the previous paper (1); the reaction mixture, consisting of 2 mM ATP, 10 mM MgCl_2 , 144 mM KCl, 0.01 mM CaCl_2 containing $82\mu\text{C}$ of Ca^{45} per mmole Ca, 20 mM Tris-maleate buffer (pH 6.8) and certain amount of vesicular fraction, was centrifuged at 40,000 r. p. m. for 15 minutes by the use of No. 40 rotor of preparative Spinco ultracentrifuge, and the resulted supernatant and residue were analyzed for their radioactivities.

Determination of Calcium Contained in Actomyosin—Actomyosin suspensions, containing no added magnesium, was deproteinized by trichloroacetic acid (final concentration, 5 percent), and the trichloroacetic acid in the solution was removed by ether extraction. Calcium in the solution was determined by the method of Yanagisawa (6).

Determination of ATPase Activity of Actomyosin—ATPase was determined by measuring the increase of inorganic orthophosphate by the method of Fiske and SubbaRow (7).

Determination of Protein Concentration—Determined as described in the previous paper (1). Otherwise noted, dry weight of protein was used.

Materials—ATP (Pabst & Co., U. S. A.); Ba-Ag salt of phosphoenolpyruvic acid and pyruvate kinase (Boe-

hringer, Germany); chelating compounds except DTTA (Dojindo, Japan). DTTA was kindly supplied by Prof. G. Schwarzenbach, to whom the author's thanks are due to.

RESULTS

Correlation between the Calcium Binding and the Relaxation Activities of Some Chelating Agents—As reported in a preliminary communication (2) there could be observed a definite correlation between the calcium binding of various chelating agents and their relaxation activities (Table I).

Effect of Calcium Ions on the Superprecipitation of Actomyosin System—Fig. 1 shows the effect of a small amount of calcium ions on the process of actomyosin superprecipitation. It is interesting that such a minute amount of calcium as $1 \times 10^{-6} M$ definitely accelerates the rate of syneresis.

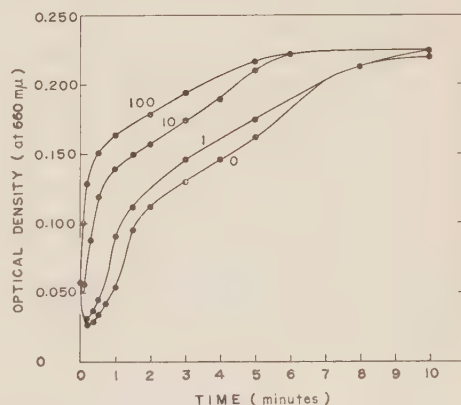


FIG. 1. Effect of calcium on actomyosin superprecipitation.

The original actomyosin sample was diluted with ten volumes of water and centrifuged; the residue was washed twice with ten volumes of 0.05 M KCl and suspended in the following solution (final concentration): 0.088 M KCl, 0.001 M MgCl_2 , 0.02 M Tris-maleate buffer (pH, 6.72, containing no alkaline metal) and a specified amount of CaCl_2 (expressed in the figure in μM). Final actomyosin concentration: 0.82 mg./ml. At zero time, 0.1 ml. of 0.04 M ATP (pH, 6.8) was added to the test solution of 4 ml. contained in the cuvette of spectrophotometer. Temperature: 23°C .

TABLE I
Relaxing and Calcium-binding Activities of Chelating Agents

	Relaxation activities in relative value ¹⁾	Calculated stability constants for Ca ²⁾	Observed stability constants for Ca ³⁾	
		$\times 10^4$	$\times 10^4$	
EDTA	(1.0)	1.38	0.89	(1.0) ⁴⁾
CyDTA	0.10	0.85	0.10	(0.11)
GEDTA	8.2	160	>40	(>45)
DTPA	0.12	1.51	0.18	(0.20)
EEDTA	0.37	0.69	0.31	(0.35)
EDTA-OH	0.25	—	0.26	(0.29)
NTA	0.05	0.093	0.07	(0.08)
DTTTA	1.0	0.32	0.91	(1.02)

1) derived from the data of the reference (5)

2) figures indicate the calculated apparent stability constants for calcium in the presence of 10 mM Mg at pH 6.8; these values are valid when the concentrations of chelating agents are between 2 mM and 0.2 mM and that of calcium are less than 0.02 mM. Formation constants and dissociation constants used in this calculation were derived from the reference (18), (19) and (20)

3) see the Materials and Methods.

4) indicate the values relative to EDTA.

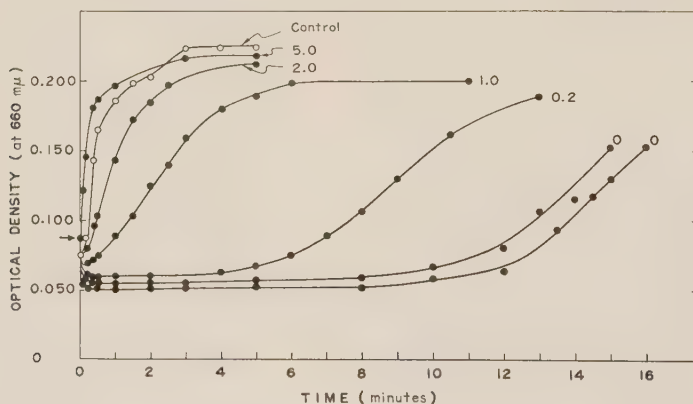


FIG. 2. Superprecipitation of EDTA-washed actomyosin and its response to calcium.

Original actomyosin sample was diluted with ten volumes of water and centrifuged; the residue was again diluted with ten volumes of 0.05 M KCl and, after centrifugation, the sediment was mixed with five volumes of 0.05 M KCl containing 0.005 M EDTA, 0.003 M MgCl_2 and 0.01 M maleate-buffer (pH 6.4). After centrifugation, the residue was washed five times with ten volumes of 0.05 M KCl by dilution and centrifuge, and then suspended in the following test solution; 0.060 M KCl, 0.001 M MgCl_2 , 0.02 M Tris-maleate buffer (pH, 6.74; containing no alkaline metal) and a specified amount of CaCl_2 (shown in the figure in μM). Control sample was treated by the same procedures described above except that EDTA was omitted from the third washing. One of two tests containing no added calcium (noted as "0" in the figure) was done at the beginning of the series of this experiment and the other, at the end. Actomyosin concentration: 0.62 mg./ml. Other experimental conditions were the same as Fig. 1. Above procedures decrease the calcium content of actomyosin preparation from 5.3 to 2.6 $\mu\text{moles per g. protein}$ (average of six trials).

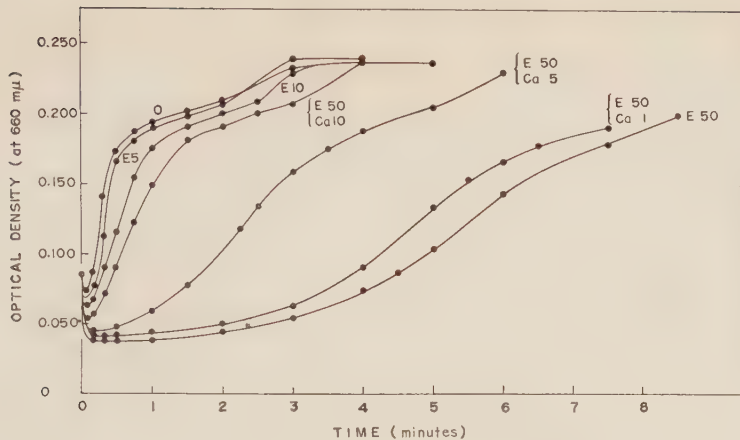


FIG. 3. Effect of calcium on the superprecipitation of actomyosin in the presence of EDTA.

The same sample as the control in Fig. 2 (not washed with EDTA) was used. Experimental conditions were the same as Fig. 2 except that the actomyosin concentration was 0.74 mg./ml. E: concentration of EDTA in μM . Ca: concentration of Ca in μM .

This result suggested to us that, if we could decrease the content of calcium in the actomyosin preparation, we might obtain such a preparation much less easily superprecipitated by ATP.

With this in mind, the actomyosin preparation was washed with EDTA following the procedures described in the legend of Fig. 2. These procedures reduced its content of calcium by half. This preparation, as shown in Fig. 2, did not make a shrinking response to ATP, unlike the control preparation, and moreover, the addition to it of a minute amount of calcium ions restored the shrinking capacity of the actomyosin preparation. It should be mentioned that a much smaller amount of calcium than in the case of Fig. 1 was sufficient to produce a significant effect on the contraction*.

One might argue that the results obtained

* During this experiment, precautions were taken to avoid the contamination of the test solution by calcium ions from any of reagents and containers. If not, we could never obtain any reproducible results. It is also to be pointed out that the actomyosin suspension tends to become more susceptible to the shrinking action of ATP with the passage of time; therefore, one series of experiments should be made within two hours.

in Fig. 2 might be due to the remaining EDTA. According to the results of a large scale experiment, there could be found practically no binding between EDTA and actomyosin gel (unpublished data). Furthermore, E. Bozler (personal communication), using glycerinated fibers, could not find any appreciable binding of EDTA with muscle fibers. It is reasonable that such polyanionic substances as EDTA might combine only with difficulty with actomyosin, a large polyanionic protein. Therefore, it is probable that the preparation used in Fig. 2, after several times washing, does not contain any significant amount of EDTA, though it is impossible to make chemical analysis of a minute amount of EDTA contained in the small amount of sample used in Fig. 2. The experiment shown in Fig. 3 provides further evidence that EDTA was not contained in the sample of Fig. 2. Addition to it of even 50 μM of EDTA could not produce the same effect as observed with the exhaustively washed sample of Fig. 2. It is not likely that such a high amount of EDTA remains in the actomyosin suspension after washing of several times. Furthermore, 1 μM of calcium did not produce any remarkable effect on the unwashed control sample to which EDTA was added (Fig 3). To

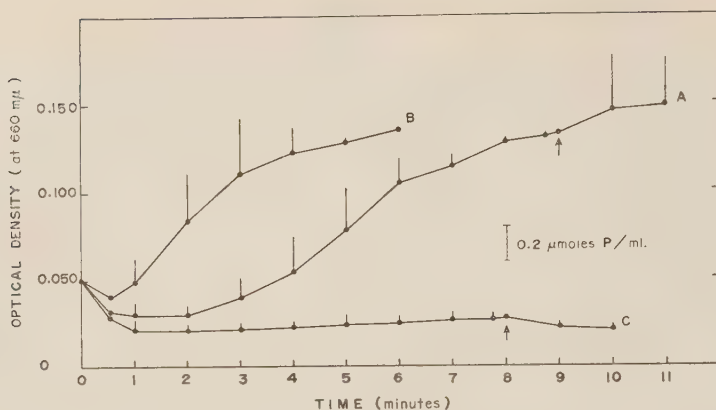


FIG. 4. ATPase activities of actomyosin during the course of superprecipitation.

The actomyosin sample was prepared by the same procedures as Fig. 1 except that: in A, 0.088 *M* KCl, in B, 0.088 *M* KCl plus 4 μ *M* CaCl_2 and in C, 0.115 *M* KCl plus 4 μ *M* CaCl_2 were used; final actomyosin concentration, 0.42 mg./ml.; total volumes of test solution, 12 ml. Every minute except the points indicated by arrow an aliquote of 0.5 ml. was taken out from the test solution and its inorganic phosphates were analyzed. At the time indicated by arrow ATP, 1 mM (final concentration), was again added. The length of perpendicular lines at every minute on every line indicates the amount of inorganic phosphates per ml. of test solutions liberated during the preceding one minute (in cases of 9th minute in A and 8th minute in C measurements were made 15 seconds prior to the appropriate time).

obtain the same effect as 1 μ *M* of calcium with EDTA washed sample, 5 μ *M* of calcium, or more, was necessary. This clearly indicates that the inhibition of syneresis in Fig. 2 is not due to the remaining EDTA.

Fig. 4 shows the correlation of the ATPase activity of actomyosin during the course of superprecipitation with the turbidity of actomyosin. In a state of decreased turbidity, *viz.*, in a relaxed state, ATPase is very low; concurrently with the onset of increase in turbidity, *viz.*, the onset of contraction, the ATPase begins to increase. After a while the apparent ATPase decreases, whereas the turbidity still increases or is kept fairly constant. This is not due to the stop of ATPase action of actomyosin, but mainly due to the exhaustion of ATP in the test solution, because if a more ATP is added at this point, a high ATPase activity again can be observed corresponding to its strong turbidity. It is to be noted that the addition of calcium would not directly influence the ATPase of actomyosin, if it did not affect the shrinking

process of actomyosin, *i. e.*, under conditions in which Ca did not produce superprecipitation, it also failed to stimulate the ATPase. These facts indicate that the ATPase of actomyosin is the expression of the physical or colloidal state of actomyosin*, *i. e.* the degree of shrunken state decides that of enzymatic activity.

Quantitative Comparison of Calcium Binding of Vesicular Relaxing Factor with that of EDTA—It is now very likely that the relaxation action of EDTA and other chelating substances is produced by removing calcium from contractile protein. We therefore attempted to make a quantitative comparison between the calcium binding capacity of EDTA and of vesicular fraction, *i. e.*, to find whether the calcium binding capacity of vesicular fraction is actually comparable with

* These results were confirmed by K. Maruyama (personal communication). A similar conclusion was reached by Tonomura and Yoshimura (8) based on the kinetic analysis of superoptimum inhibition of actomyosin ATPase.

that of EDTA and its analogues.

Previous paper (1) has shown that, the stability constant between calcium and vesicular fraction is approximately 0.8×10^5 in the absence of myokinase or 1.2×10^5 in the presence of myokinase. This is a striking value, because the experiments, shown in Table I, revealed that the stability constants between calcium and EDTA under the similar conditions was only about 10^4 .

This strong affinity of vesicular fraction for calcium was verified by another experiment (Fig. 5). This shows that the calcium binding of the vesicular relaxing factor in the

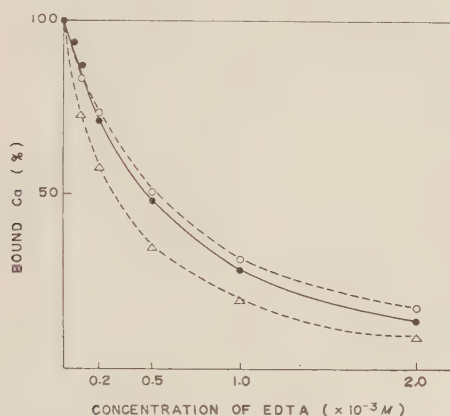


FIG. 5. Calcium binding of vesicular fraction in the presence of EDTA.

The ordinate shows values relative to the maximum binding of calcium by the vesicular fraction in the presence of $0.01 M$ $CaCl_2$ (the details of the procedures were described in the previous paper (1)). Two mg. (wet weight) of vesicular fraction per ml. were used. Calculations were made under the assumptions: stability constants with calcium and maximum calcium binding of vesicular fraction, 10^5 and $10 \mu moles/g.$ (wet weight) respectively; apparent stability constants of EDTA under the present conditions, 0.5×10^4 for upper curve (O), and 1×10^4 for lower curve (Δ) (see Table I). ●, observed.

presence of EDTA is strong enough to successfully compete with EDTA for calcium, unless the concentration of EDTA is raised to high levels. Furthermore, the curve for

calcium binding in the presence of increasing amounts of EDTA, calculated from the independently determined stability constants, agrees with the experimental curve.

We then compared the relaxing effects of chelating substances and vesicles on the basis of their calcium binding. Fig. 6 shows the relaxing effect of both agents on the same preparation of actomyosin. The results show that 0.42 mg. (wet weight) per ml. of relaxing vesicles corresponded to about $40 \mu M$ EDTA. It is to be pointed out here that vesicular relaxing factor is effective on extracted actomyosin. This confirms Müller's view (9) derived from the results with his centrifuge method that the oriented structure of actomyosin, such as myofibril or glycerinated muscle fibers, is not essential for the action of vesicular relaxing factor.

It is also important to observe whether the actual removal of calcium from actomyosin by relaxing vesicles surely corresponds to that by chelating agents. Table II shows the calcium depriving activity of relaxing vesicles, EDTA and GEDTA. The relative activities among those agents again correspond well with the results described above, especially those of Table I. Roughly speaking, 3.3 mg. of vesicles (wet weight) per ml. corresponds to $0.7 mM$ EDTA. If we take the different experimental conditions into consideration, these values are in good agreement with the result shown in Fig. 6.

It should be pointed out that a low concentration of ATP was used in this experiment. As described before, the calcium binding activity of vesicular fraction is dependent on ATP. If the ATP concentration is high, it would dissolve actomyosin even in the solution of low ionic strength and leaves some protein in the supernatant even after a strong centrifugation. To avoid these troubles, a low concentration of ATP was used supplemented with the phosphoenolpyruvate-pyruvate kinase system. Under these conditions no appreciable protein was found in the supernatant except the case containing vesicles.

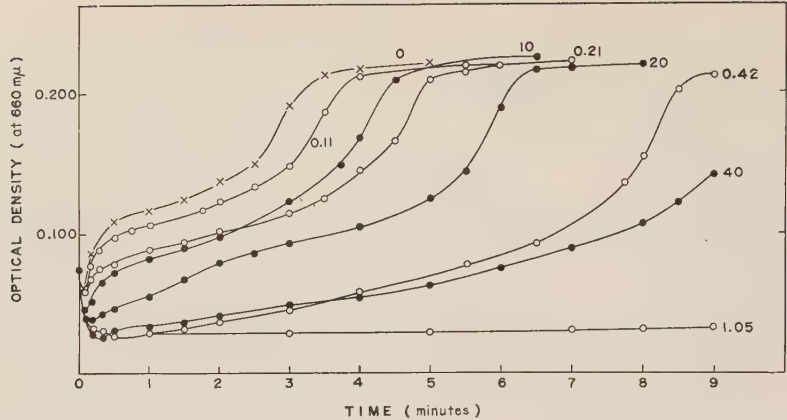


FIG. 6. Relaxation activity of vesicular relaxing factor compared with that of EDTA.

The original actomyosin sample was treated in the same way as described for Fig. 1. Experimental conditions were the same as Fig. 1 except that 0.072 *M* KCl and 0.91 mg./ml. of actomyosin were used. Concentration of EDTA (●) and vesicles (○) were expressed in μ M and mg. (wet weight) per ml. respectively. $\times$, control.

TABLE II

Removal of Calcium from Actomyosin with Chelating Compounds and Vesicular Fraction

	Ca ⁴⁵ in the residue	Ca ⁴⁵ in the supernatant
	c.p.m.	c.p.m.
Control	4,280 (51%)	4,160
EDTA 0.5 mM	3,050 (37%)	5,480
EDTA 1 mM	1,860 (22%)	6,520
GEDTA 0.5 mM	855 (10%)	7,680
Vesicular fraction 3.3 mg./ml.	2,580 (31%)	5,760

Original actomyosin was diluted with ten volumes of water and centrifuged; the residue was suspended in 0.05 *M* KCl solution, to which 0.1 μ M CaCl₂ containing Ca⁴⁵ (final concentrations were indicated) was added. After one hour, it was centrifuged and the residue was washed four times with ten volumes of 0.05 *M* KCl. The residue was suspended to make solution of 5 ml. (figures indicate final concentration): 0.05 *M* KCl, 1 mM MgCl₂, 0.02 *M* Tris-maleate buffer (pH, 6.8; contained no alkaline metal), 5 mM phosphoenolpyruvate, 10 units/ml. pyruvate kinase, a specified amount of agents described above and 0.1 mM ATP (ATP was added at last). Actomyosin concentration: 1.3 mg./ml. Immediately after addition of ATP the test solution was centrifuged at

4,000 $\times$ *g* for 10 minutes, at which gravity vesicles stayed up while actomyosin was precipitated. Both supernatant and residue were analyzed for their radioactivities due to Ca⁴⁵. Counts of radioactivity was corrected to infinite thinness.

DISCUSSION

Results reported in the previous paper (1, 2) have already suggested an intimate correlation between the calcium binding activity of the vesicular fraction of skeletal muscle and its relaxing activity. For example, both activities are easily inactivated by aging for a few days and by a low concentration of detergents; moreover, these activities are not found in the similar vesicular fraction of other tissues. This suggestion was further substantiated in the present paper. It was shown that the relaxation by chelating agents was due to their removal of calcium from the contractile element, and that the calcium-binding capacity of the vesicular relaxing fraction was strong enough to be compared with these chelating substances. Furthermore, calcium was shown to be essential for the contraction of actomyosin system, in agreement with the interesting work of A. Weber, (10) based on her enzymatic studies, and also with the earlier suggestion of Bozler (11).

We now believe that it is the calcium binding action itself of the vesicles which is the mechanism of the relaxing action.

The important role of calcium in the contraction of living muscle has long been supposed by some physiologists. Heilbrun (12) has demonstrated that calcium is the only substance of physiological origin which, when injected into the interior of a cell, can produce contraction. Shanes (13) has postulated that calcium is a trigger substance in initiating the muscle contraction. Our experiments carried out at the subcellular level with isolated cellular components appear to lend positive support to the earlier views of those physiologists implicating calcium.

As described in the previous paper, the relaxing factor is probably identical as parts of endoplasmic reticulum. The vesicular structure of the factor was also noted by Nagai, Makinose and Hasselbach (21). It has been suggested by several workers, e.g., Bennett and Porter (14), Porter and Palade (15), and A. F. Huxley and Taylor (16, 17), that the endoplasmic reticulum might represent a mean by which the excitation of the sarcolemma is conducted inwards to the interior of the muscle fiber. The morphological basis of the relaxing factor mentioned above lends further support to the involvement of the endoplasmic reticulum in the mechanism linking excitation and contraction.

Considering these suppositions, especially of Huxley and Taylor, and the result described in the present paper, we suggest the following picture of muscle contraction. In resting muscle calcium is highly concentrated in the endoplasmic reticulum; the concentration of calcium inside the myofibril is too low to cause the shrinking of contractile protein. When the muscle is excited, the concentrated calcium in some portion of the endoplasmic reticulum is released by the electrical influence due to the depolarization of the muscle membrane, and the calcium thus released causes in turn the shrinking of the actomyosin system. When the excitation is over, calcium release ceases and the liberat-

ed calcium is recaptured by the endoplasmic reticulum (the relaxing factor); consequently, the shrunken actomyosin-system is restored to its relaxed state. This picture is compatible with the facts in living muscle observed by physiologists, but so far it is supported by rather few evidences. Further investigation is needed which are now in progress.

SUMMARY

1. A minute amount of calcium is essential for actomyosin superprecipitation.
2. Relaxation activities of some chelating agents have a definite correlation with their calcium binding activities.
3. ATP-dependent calcium binding activity of vesicular relaxing factor has a capacity comparable to that of the chelating agents. This accounts for the relaxing activity of the factor.
4. The role of the vesicular relaxing factor, presumably identical with some part of the endoplasmic reticulum, in physiological process of muscular contraction was discussed. The possible role of calcium in the mechanism linking excitation and contraction was suggested.

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Isolation and Characterization of a New Enzyme Choline Sulfatase

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Since the first description of the occurrence of choline sulfate in the mycelium of *Aspergillus sydowi* (1), numerous species of fungi have been found to contain this sulfate ester (2, 3). A remarkably high content of this compound in the conidiospores of *Aspergillus niger* was reported recently by the present author and the significance of this substance as a reservoir of sulfur was suggested (4). Investigation of the biosynthesis of choline sulfate has revealed a mechanism involving sulfate transfer from adenosine-3'-phosphate-5'-phosphosulfate to choline (5). Earlier investigators have found, on the other hand, that this sulfate ester is not hydrolyzed by any of the known sulfatases (6) or by cholinesterase (7). Attempts to detect the reversal of the biosynthetic reaction of choline sulfate in a cell-free system have also been unsuccessful (5). Thus, in spite of the well known fact that choline sulfate serves as a good source of sulfur for the growth of various fungi (8), the mode of its utilization has been quite obscure.

The present report deals with the isolation from a soil bacterium of an enzyme which specifically hydrolyzes choline sulfate. Investigation of some properties of the partially purified preparation led to the conclusion that this enzyme represents a new species of sulfatase for which the name "choline sulfatase" is proposed.

MATERIALS AND METHODS

Isolation of Organism—For the isolation of microorganisms capable of active utilization of choline sulfate, a synthetic medium was prepared which contained choline sulfate as the sole source of carbon, nitrogen and sulfur. The composition of the isolation medium was as follows: choline sulfate, 5 g.; KH_2PO_4 ,

1 g.; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g.; trace-element solution*, 1 ml.; distilled water to 1 liter. Using this medium, eight strains of rod-shaped bacteria and one strain of a yeast-like organism were isolated from soil collected in the vicinity of this Institute. Among these organisms, one bacterial strain was selected and used throughout this study, because it showed the highest activity in the utilization of choline sulfate. This organism was a Gram-negative short rod with a polar flagellum, and was identified by Dr. Kazuo Komagata of this Institute as *Pseudomonas nitroreducens* Iizuka et Komagata (9). An electron micrograph of this bacterium is shown in Fig. 1.

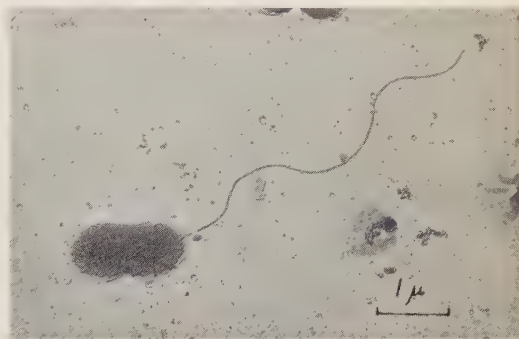


FIG. 1. Electron micrograph of *Pseudomonas nitroreducens* grown on choline sulfate as the sole organic substrate.

Cultivation of Organism—For studies of the growth characteristics and to prepare the enzyme, the organism was cultivated at 30°C with vigorous shaking in a synthetic medium of the following composition: choline sulfate, 1.83 g.; KH_2PO_4 , 2.71 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 4.36 g.; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$,

* The trace-element solution had the following composition: $\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$, 4 g.; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 970 mg.; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 300 mg.; $\text{MgCl}_2 \cdot 4\text{H}_2\text{O}$, 72 mg.; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 37 mg.; $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 88 mg.; distilled water to 1 liter.

0.1g.; trace-element solution (as specified above), 1ml.; Tween 80, 0.1ml.; distilled water to 1 liter. A fairly large amount of phosphates was included in this medium in order to prevent the pH from being lowered by the sulfuric acid liberated as growth proceeds. Under these conditions of culture the organism multiplied with an average doubling time of 110 minutes, and attained a maximum growth of 450-500mg. dry weight of cells per liter.

Assay of Enzyme Activities—Unless otherwise specified, the enzyme reaction was carried out at 37°C in 0.02 *M* tris buffer (pH 8.3) with a substrate concentration of 0.01 *M*. The enzyme activity of choline sulfate hydrolysis was usually assayed by determining the amount of sulfate ion liberated in an appropriate period, but in some experiments the activity was estimated by determining the free choline. Sulfate ion in the reaction mixture was determined by a modification of the barium chloranilate method of Lloyd (10). To 0.5ml. of a reaction mixture were added 2ml. of ethanol and 1ml. of 0.25 *M* acetate buffer (pH 4.0) and finally 0.5ml. of a suspension of barium chloranilate (1mg./ml.) in 0.25 *M* acetate buffer (pH 4.0). The mixture was shaken vigorously at intervals and after not less than 20 minutes centrifuged to remove excess barium chloranilate and precipitated barium sulfate. The amount of chloranilate ion released into solution was measured spectrophotometrically at 327.5 $m\mu$ against a reagent blank. Choline determination was performed according to the procedure of Hayashi *et al.* (11). To 1ml. of a sample containing choline were added 0.2ml. of 0.5 *M* acetate buffer (pH 5.0), 0.1ml. of aqueous solution of KI (0.1 *M*) and then 4ml. of 0.5% I_2 solution in ethylene dichloride. The mixture was shaken vigorously for 10 seconds and centrifuged to give clear separation of the aqueous and solvent layers. The optical density of the solvent layer was read at 385 $m\mu$ against a reagent blank. In these determinations of sulfate and choline, it was found unnecessary to deproteinize the reaction mixture before making the determinations. Enzyme activity was expressed as μ moles of choline sulfate hydrolyzed per ml. in one hour.

To study the stoichiometric relationship of the reaction, the decrease in amount of choline sulfate was estimated, using S^{35} -labeled substrate. In this case the reaction was stopped by the addition of 0.02 *N* HCl to a final concentration of 0.005 *N*, and the $S^{35}O_4^{--}$ was precipitated by adding an appropriate amount of $BaCl_2$ and carrier sulfate. The changes in the amounts of choline sulfate and inorganic sulfate were calculated from the changes in the radioactivity

of supernatant solution and precipitate, respectively. Radioactivity measurements were performed on samples dried in steel planchets, using an end-window type Geiger counter. Self absorption was negligible under the conditions used, and the values were corrected for background counts.

Aryl sulfatase activity was estimated using *p*-nitrophenyl sulfate and nitrocatechol sulfate as substrates, by determining the amount of *p*-nitrophenol and nitrocatechol liberated according to the method of Webb and Morrow (12). Sulfatase activity toward other sulfate esters was assayed by the determination of inorganic sulfate liberated according to the method mentioned above. Cholinesterase activity was assayed by measuring the changes in the amount of remaining substrate, acetylcholine, according to the procedure of Hestrin (13). Hydrolysis of phosphorylcholine was studied by measuring the amount of choline liberated according to the method described above.

Paper Electrophoresis and Paper Chromatography—The products of choline sulfate hydrolysis were identified by paper electrophoresis, using Toyo No. 50 paper with 0.05 *M* phosphate buffer (pH 7.0) as an electrolyte solution at a potential gradient of 12 volt./cm. Under these conditions sulfate ion, choline sulfate and choline moved toward the anode +7.0, -0.5 and -4.5 cm., respectively, in 2 hours. Choline was also identified by paper chromatography, using a solvent system of *sec*-butanol-formic acid-water (75:15.5:14.5). The R_f values of choline sulfate and choline were 0.28 and 0.37, respectively. Inorganic sulfate and choline sulfate were detected by radioautography, using S^{35} -labeled samples; choline was detected by exposing the paper to I_2 vapor.

Materials—Choline sulfate was synthesized according to the directions of Schmidt and Wagner (14). S^{35} -Choline sulfate was prepared according to the procedure of Stevens *et al.* (15), and used at the specific activity of 282 c.p.m./ μ mole. *p*-Nitrophenyl sulfate was obtained from Sigma Chemical Co. The other sulfate esters were kindly provided by Dr. N. Takahashi of Nagoya University. Phosphorylcholine was obtained through courtesy of Dr. M. Hayashi of Chiba University. The Takadiastase used was a commercial sample.

RESULTS

Extraction and Partial Purification of Enzyme—Preliminary experiments showed that the intact or lyophilized cells of *Pseudomonas nitroreducens* grown on choline sulfate exhibited a remarkable cleaving activity with respect to

this sulfate ester. The activity was not detected, on the other hand, in the growth medium from which the cells were harvested. The enzyme responsible for the breakdown of choline sulfate was, therefore, extracted from the cells of this organism growing actively in the synthetic medium described in Materials and Methods. Cells (2.52 g. dry weight) harvested from 5 liters of a 22-hour culture were washed twice with 0.2 *M* phosphate buffer (pH 6.6) and suspended in 20 ml. of 0.02 *M* phosphate buffer (pH 6.6). The thick suspension of cells thus obtained was subjected to sonic oscillation (10 KC) for 7 minutes and then centrifuged at $100,000\times g$ for 60 minutes in order to remove cell debris and a particulate fraction, which were found to show no hydrolytic activity toward choline sulfate. The cell-free sonicate thus obtained, as well as lyophilized cells, was used in some experiments as the enzyme preparation, but for most of the experiments described below the enzyme was partially purified by means of fractional precipitation with ammonium sulfate. It was preliminarily shown that the enzyme activity is contained in the fractions precipitable at low concentrations of ammonium sulfate. Therefore, starting from the above sonicate, fractionation was repeated four times with ammonium sulfate concentrations ranging from 11.4 to 35.5 g. in 100 ml. In each step of fractionation, the precipitates were dissolved in distilled water and dialyzed overnight at 4°C against 0.005 *M* phosphate buffer (pH 6.6). The preparation with highest specific activity finally obtained was the fraction which precipitated at ammonium sulfate concentrations between 16.0 and 22.0 g. in 100 ml. Such a preparation contained 10 per cent of the total activity and 2.4 per cent of the protein (measured as optical density at 280 *mμ*) originally found in the sonicate. Any of other fractions did not give marked activity. The specific activity thus increased only four-fold after the purification procedures. This seems to reflect, at least partially, the instability of the enzyme during the course of fractionation, since recovery of the activity in each fractionation step was rather low

(37.5–82 per cent). No further attempt was made to purify the enzyme, and this fraction was used as the enzyme preparation for the characterization of the enzyme. One ml. of this preparation contained 21 mg. of protein (measured gravimetrically) and hydrolyzed 90 μ moles of choline sulfate in one hour under optimal conditions. The preparation was stable for at least two months when adjusted to pH 6.6 by citrate or phosphate buffer and stored at -20°C .

Qualitative Analysis of Reaction Products—The products of the enzymatic breakdown of choline sulfate were first studied qualitatively. In this experiment S^{35} -choline sulfate was incubated with lyophilized cells or unfractionated sonic lysate of *Pseudomonas nitroreducens* for 6 hours in 0.025 *M* tris buffer (pH 7.4). The reaction was stopped by heating the reaction mixture in a boiling water bath for 2 minutes and after the removal of insoluble matter by centrifugation, the supernatant solution was concentrated to a small volume under reduced pressure. This sample was then spotted on filter paper and subjected to paper electrophoretic analysis. Fig. 2 illustrates the results of this experiment, which clearly showed that choline and inorganic sulfate were liberated in this system. The choline formed in the reaction mixture was further identified by means of paper chromatography as described in Materials and Methods.

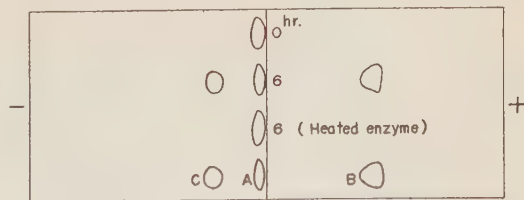


FIG. 2. Identification of reaction products by paper electrophoresis. A: S^{35} -choline sulfate, B: $\text{S}^{35}\text{O}^{--}_4$, C: choline.

Stoichiometry of the Reaction—The stoichiometric relationship between the substrate and the products was studied using S^{35} -choline sulfate as substrate and lyophilized cells as enzyme preparation. The disappearance of

choline sulfate was estimated from the decrease in the radioactivity of the supernatant obtained after the addition of BaCl_2 to the reaction mixture. The amount of inorganic sulfate liberated was estimated colorimetrically and also from the increase in the radioactivity of the precipitate formed with BaCl_2 . Choline liberation was measured colorimetrically. The results of the analysis shown in Table I indicate that one mole of choline sulfate is hydrolyzed by the action of the enzyme, resulting in the formation of one mole of choline and one mole of inorganic sulfate, as shown in the following formula:

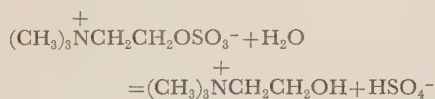


TABLE I

*Stoichiometry of Hydrolysis of Choline Sulfate
by Pseudomonas Enzyme*

Substance	Balance sheet in reaction system ($\mu\text{mole/ml.}$)
Choline sulfate	-2.73 ¹⁾
Sulfate	+2.26 ¹⁾ +2.41 ²⁾
Choline	+2.74 ²⁾

- 1) Estimated from change in radioactivity.
- 2) Determined colorimetrically.

Lyophilized cells of *Pseudomonas nitroreducens* were incubated with 0.01 M S^{35} -choline sulfate (282 c.p.m./ μmole) at 37°C for 4 hours in 0.01 M tris buffer (pH 8.3).

Heat- and pH-Stability of Enzyme—The partially purified enzyme preparation was adjusted to pH 6.6 with 0.005 M citrate buffer, and maintained at various temperatures for 10 minutes. After the treatment the preparation was immediately brought to 37°C and comparison was made of the activity of the preparations with and without heat treatment. The results shown in Table II indicate that treatment at 40°C for 10 minutes decreased the activity by 29 per cent. Treatment for 10 minutes at temperatures higher than 60°C completely inactivated the enzyme.

The stability of the partially purified

preparation at various pH's was examined by diluting the preparation with 0.005 M buffers of various pH's and keeping these preparations at 4°C for 10 minutes. After the treatment, the pH of each preparation was promptly adjusted to 8.3 by the addition of an appropriate amount of 0.05 M tris buffer (pH 8.3) and the activity of the preparations was measured under the optimal conditions. This experiment showed that the enzyme was

TABLE II

Effect of Heat on Activity of Pseudomonas Enzyme

Temperature of treatment	Activity ($\mu\text{mole SO}_4$ liberated/ml./hr.)
No treatment	1.02
30°C	1.02
40°	.71
60°	.00
80°	.00
100°	.00

Heat treatment applied for 10 minutes at pH 6.6. Reaction was carried out at 37°C for 30 minutes with 0.02 M substrate and in 0.02 M tris buffer (pH 8.3). Final concentration of enzyme in reaction mixture was 0.21 mg. protein/ml.

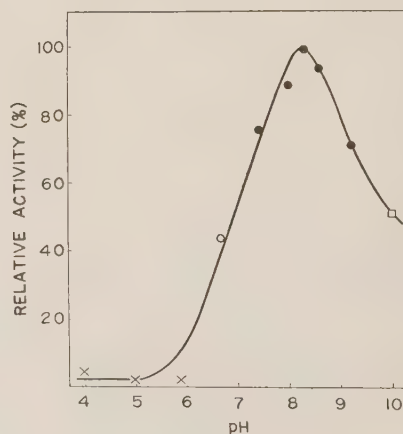


FIG. 3. pH-Activity curve for the hydrolysis of choline sulfate by *Pseudomonas* enzyme.

Lyophilized cell preparation was incubated with 0.01 M choline sulfate for 60 minutes at 37°C. Reaction was carried out in 0.01 M acetate buffer (x), citrate buffer (○), tris buffer (●), and glycine buffer (□).

most stable at pH 6.6 and was rapidly inactivated at lower pH's; 91% inactivation occurred after 10 minutes in citrate buffer of pH 4.2.

Effect of pH and Substrate Concentration on Reaction Rate—The effect of variation of pH on the rate of enzymatic hydrolysis of choline sulfate is shown in Fig. 3. Maximal activity was observed at pH 8.3 and the reaction proceeded, if at all, only very slowly at pH's under 6.0. This may be due to the inactivation of the enzyme at lower pH's.

The effect of substrate concentration on the reaction rate was also studied using the partially purified enzyme preparation. Fig. 4 shows the Lineweaver-Burke plot of the results obtained. The Michaelis constant was estimated to be $4 \times 10^{-2} M$ under the present experimental conditions (pH 8.3, 37°C).

Effect of Certain Inhibitors—In an effort to characterize the enzyme concerned in the hydrolysis of choline sulfate, studies were carried out on the behavior of the partially purified preparation toward certain inhibitors of known sulfatases and also toward those of cholinesterase.

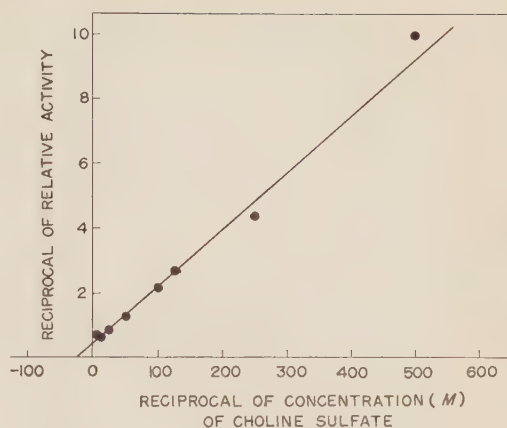


FIG. 4. Effect of substrate concentration on rate of enzymatic hydrolysis of choline sulfate.

Concentrations in reaction mixture: partially purified enzyme preparation, 0.42 mg. protein/ml.; tris buffer (pH 8.3), 0.02 *M*. Reaction was carried out at 37°C for 60 minutes.

The effect of several inhibitors of sulfatases on the enzymatic hydrolysis of choline sulfate is shown in Table III. As can be seen in the Table the activity of the enzyme was marked-

TABEL III

Effect of Certain Inhibitors of Sulfatases on Enzymatic Hydrolysis of Choline Sulfate

Addition	Concentration (M)	Activity (% of control)	Addition	Concentration (M)	Activity (% of control)
Na ₂ SO ₃	1 × 10 ⁻⁶	99	Na ₂ HPO ₄	1 × 10 ⁻⁴	92
	1 × 10 ⁻⁵	67		3 × 10 ⁻⁴	104
	1 × 10 ⁻⁴	17		1 × 10 ⁻³	111
	1 × 10 ⁻³	2		3 × 10 ⁻³	118
	1 × 10 ⁻²	1		1 × 10 ⁻²	107
K ₂ SO ₄				3 × 10 ⁻²	116
	1 × 10 ⁻³	104	KCN	1 × 10 ⁻³	149
	3 × 10 ⁻³	99		3 × 10 ⁻³	145
	1 × 10 ⁻²	117		1 × 10 ⁻²	128
	3 × 10 ⁻²	106		3 × 10 ⁻²	80
NaF					
	1 × 10 ⁻⁴	102			
	1 × 10 ⁻³	90			
	1 × 10 ⁻²	88			

In experiments with Na₂SO₃ and K₂SO₄, activity was assayed by determination of choline liberated; in other cases, by determination of sulfate liberated. Concentrations in reaction mixture: choline sulfate, 0.01 *M*; partially purified *Pseudomonas* enzyme, 0.21 mg. protein/ml.; tris buffer (pH 8.3), 0.02 *M*. Reaction carried out at 37°C for 60 minutes.

ly inhibited by sulfite, one of the strong inhibitors of aryl sulfatases, the concentration of 50% inhibition being $2.5 \times 10^{-5} M$. It was rather surprising, on the other hand, that the rate of hydrolysis of choline sulfate was not affected by sulfate, phosphate, fluoride and cyanide, since all the sulfatases of known type are inhibited at least by either one of these anions (16).

The effect of two inhibitors of cholinesterase on the hydrolysis of choline sulfate is shown in Table IV. Prostigmine and eserine are known to inhibit cholinesterase by competing with acetylcholine at the active site on the enzyme protein. Results in Table IV show, however, that these agents had entirely no effect on the hydrolysis of choline sulfate even at much higher concentrations than those used for cholinesterase. This finding seems to suggest that the active site of choline sulfate hydrolyzing enzyme is of different nature from that of cholinesterase.

TABLE IV

Effect of Prostigmine, Eserine and Choline on Enzymatic Hydrolysis of Choline Sulfate

Addition	Concentration (M)	Activity (% of control)
Prostigmine	1×10^{-5}	99
	1×10^{-4}	104
	4.5×10^{-4}	100
Eserine	1×10^{-5}	98
	1×10^{-4}	100
	1×10^{-3}	100
Choline chloride	1×10^{-3}	102
	1×10^{-2}	51

Concentrations in reaction mixture: choline sulfate, 0.01 M; partially purified *Pseudomonas* enzyme, 0.42 mg. protein/ml.; tris buffer (pH 8.3), 0.02 M. Reaction carried out at 37°C for 60 minutes.

Table IV also shows that choline, one of the products of the present reaction, did not show any inhibitory effect at the concentration of $1 \times 10^{-3} M$, but at a higher concentration ($1 \times 10^{-2} M$) some inhibition was observed.

Experiments were also carried out to study the effects of some divalent cations on

the enzymatic hydrolysis of choline sulfate. In these experiments the activity of the enzyme was assayed by the determination of the choline liberated, since most of the cations used are known to interfere with the determination of sulfate by the method used in this study (10). The results of a typical experiment are shown in Table V. It was found that the hydrolysis of choline sulfate by the enzyme was completely inhibited by the presence of Zn^{++} , Cu^{++} , Hg^{++} and Co^{++} at a concentration of 0.01 M, while Ba^{++} exhibited no inhibitory effect at the same concentration. Mg^{++} , Mn^{++} and Ca^{++} were found to be somewhat inhibitory.

TABLE V

Effect of Divalent Cations on Enzymatic Hydrolysis of Choline Sulfate

Metal salt (0.01 M)	Activity (μ mole choline liberated/ml./hr.)	(% of control)
No addition	1.56	100
$BaCl_2$	1.81	116
$CaCl_2$	1.23	79
$MgCl_2$	.43	28
$MnCl_2$	.71	46
$ZnCl_2$	.02	1
$CuCl_2$	.00	0
$HgCl_2$	.00	0
$CoCl_2$	.05	3

Concentrations on reaction mixture: choline sulfate, 0.01 M; partially purified *Pseudomonas* enzyme, 0.42 mg. protein/ml.; tris buffer (pH 8.3), 0.02 M; Metal chlorides, 0.01 M. Reaction carried out at 37°C for 60 minutes.

Activity toward Other Substrates—The results of these inhibition experiments suggested that the hydrolysis of choline sulfate might be catalyzed by a sulfatase of new type. However, the relatively low affinity of the enzyme preparation toward choline sulfate ($K_m = 4 \times 10^{-2} M$) was suggestive, on the other hand, of the possibility that some known sulfatase of rather broad specificity might be catalyzing the reaction. This possibility was examined directly by investigating the activity of the partially purified preparation toward sulfate

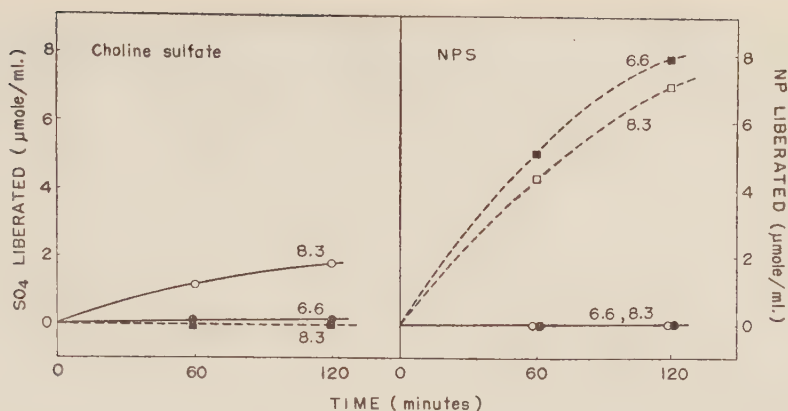


FIG. 5. Hydrolysis of choline sulfate and *p*-nitrophenyl sulfate (NPS) by *Pseudomonas* enzyme and by Takadiastase.

Concentrations in reaction mixture: choline sulfate or NPS, 0.01 *M*; partially purified *Pseudomonas* enzyme (—), 0.42 mg. protein/ml., or Takadiastase (---), 10 mg./ml.; tris buffer (pH 8.3) (○, □) or citrate buffer (pH 6.6) (●, ■), 0.02 *M*. Reaction was carried out at 37°C.

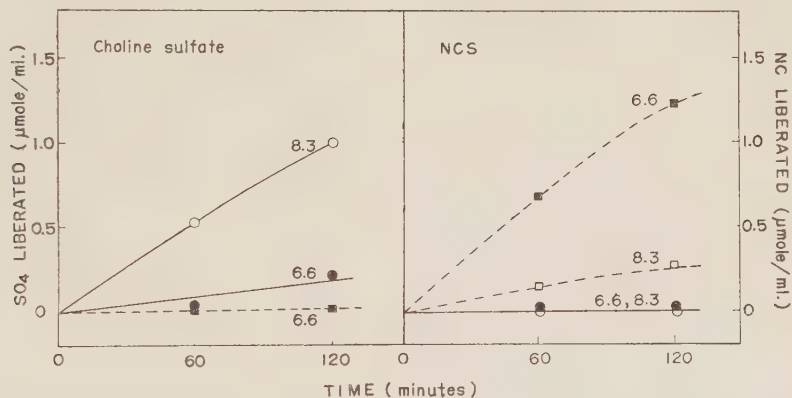


FIG. 6. Hydrolysis of choline sulfate and nitrocatechol sulfate (NCS) by *Pseudomonas* enzyme and by Takadiastase.

Concentrations in reaction mixture: choline sulfate or NCS, 0.005 *M*; partially purified *Pseudomonas* enzyme (—), 0.42 mg. protein/ml., or Takadiastase (---), 5mg./ml.; tris buffer (pH 8.3) (○, □), or citrate buffer (pH 6.6) (●, ■), 0.02 *M*. Reaction was carried out at 37°C.

esters of various kinds. In all the experiments of this type the activity was tested at two pH's: at the optimal pH for choline sulfate hydrolysis and also at the pH which is known to be favorable for the hydrolysis of the sulfate ester under study. Figs. 5 and 6 illustrate the results obtained with two phenol sulfates, *p*-nitrophenyl sulfate and nitrocatechol sulfate, respectively. It was clearly demonstrated that the preparation possesses no activity toward the phenol sulfates tested, even under

conditions which permit an active hydrolysis of these esters by the corresponding aryl sulfatases in Takadiastase.

Similar results obtained with sulfate esters of other groups are summarized in Table VI. With the single exception of amylosepolysulfate, which appeared to be slightly hydrolyzed, no sulfate ester other than choline sulfate was attacked by this enzyme preparation. It should be mentioned that unfractionated sonic lysate of this organism

TABLE VI

Activity of *Pseudomonas* Enzyme toward Substrates of Known Sulfatases

Substrate	Activity ($\mu\text{mole SO}_4$ liberated/ml./hr.)	
	at pH 5.7	at pH 8.3
Choline sulfate	.08	.96
Glucose-6-sulfate	.01	.01
Ethyl sulfate	.00	.00
Sinigrin	.00	.00
Cellulosepolysulfate (S content 18.0%)	.00	.02
Amylosepolysulfate (S content 15.8%)	.03	.08
Chondroitin sulfate (S content 6.0%)	.02	.02

Concentrations in reaction mixture: substrate, 0.01 *M*; partially purified *Pseudomonas* enzyme, 0.42 mg. protein/ml.; tris buffer (pH 8.3), 0.02 *M* or acetate buffer (pH 5.7), 0.04 *M*. For polysulfates, substrate concentration given in terms of 0.01 *M* sulfur. Reaction carried out at 37°C for 60 minutes.

also failed to exhibit any activity toward these sulfate esters. These findings were interpreted as excluding the possibility that some known sulfatase may be participating in the hydrolysis of choline sulfate by this preparation. At the same time, the results described here indicate that the choline sulfate hydrolyzing enzyme of *Pseudomonas* has a high degree of substrate specificity.

Experiments of the same type were also performed with other choline esters. Under the standard conditions of choline sulfate hydrolysis, no breakdown of acetylcholine was observed with the partially purified preparation. The absence of cholinesterase activity was further confirmed under the conditions which are usually employed for the study of cholinesterase (Fig. 7). These results, together with the fact that the activity of the enzyme is not affected by cholinesterase inhibitors, indicate that the hydrolysis of choline sulfate involves a different mechanism from that of acetylcholine hydrolysis.

Negative results were also obtained with phosphorylcholine, as shown in Fig. 8.

Adaptive Nature of Enzyme Formation—*Pseudomonas nitroreducens* was found to grow in the

synthetic medium described above even when choline sulfate was replaced by choline chloride plus inorganic sulfate in a corresponding concentration. An attempt was therefore made to determine whether or not the enzyme

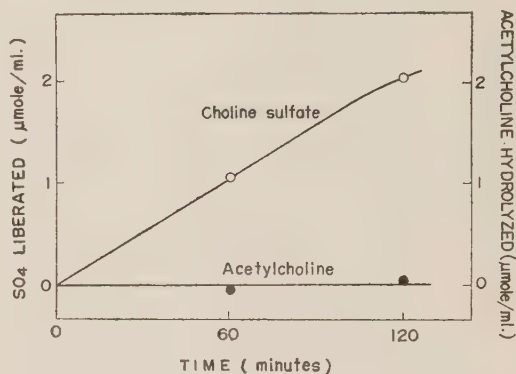


FIG. 7. Comparison of activity of *Pseudomonas* enzyme toward choline sulfate with that toward acetylcholine.

Concentrations in reaction mixture: choline sulfate or acetylcholine, 0.038 *M*; partially purified *Pseudomonas* enzyme, 1.13 mg. protein/ml.; phosphate buffer (pH 7.65), 0.023 *M*. Reaction carried out at 37°C.

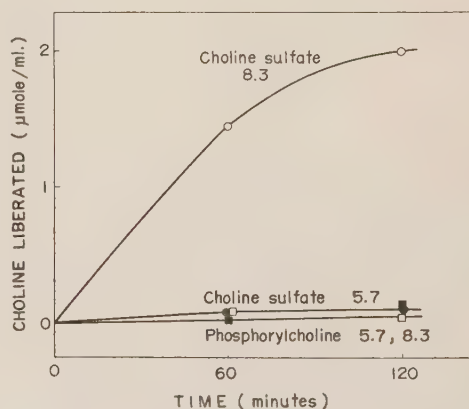


FIG. 8. Comparison of activity of *Pseudomonas* enzyme toward choline sulfate with that toward phosphorylcholine.

Concentrations in reaction mixture: choline sulfate or phosphorylcholine, 0.01 *M*; partially purified *Pseudomonas* enzyme, 0.42 mg. protein/ml.; tris buffer (pH 8.3), 0.02 *M* (○, □), or acetate buffer (pH 5.7), 0.04 *M* (●, ■); MgCl_2 (only for phosphorylcholine), 0.005 *M*. Reaction carried out at 37°C.

is formed in the absence of its substrate, choline sulfate. Growth studies of the organism using the replacement culture technique showed that, when the cells growing exponentially in a choline sulfate medium were transferred into choline chloride plus inorganic sulfate medium, they were able to grow without any appreciable lag period and even more rapidly (average doubling time of 90 minutes). Approximately one hour of lag period was observed, on the other hand, when cells were transferred from a choline chloride plus sulfate medium to a choline sulfate medium, suggesting the adaptive formation of choline sulfate hydrolyzing system. More direct evidence for the adaptive nature of the enzyme formation was obtained from the measurement of the choline sulfate hydrolyzing activity of cells which were grown in a choline chloride plus sulfate medium. The results shown in Table VII show that a

TABLE VII

Choline Sulfate Hydrolyzing Activity of Cells Grown in the Presence and Absence of Substrate

C and N source	Culture age (Hours after inoculation)	Activity (μ mole SO_4 liberated/ml./hr./mg. cell dry weight)
Choline sulfate	22	.9
	40	1.0
Choline chloride	22	.02
	40	.00

Lyophilized cell preparation incubated at 37°C for 60 minutes with 0.01 *M* choline sulfate in 0.01 *M* tris buffer (pH 8.3). Conditions of culture, see text.

lyophilized preparation of these cells contained no activity toward choline sulfate, while a corresponding preparation of cells grown on choline sulfate possessed powerful enzyme activity.

DISCUSSION

The present study establishes that a strain of *Pseudomonas* has the capacity to form adaptively an enzyme which catalyzes the hydrolysis of choline sulfate into choline and

inorganic sulfate. Although this enzyme was not extensively purified, experiments were performed with a partially purified preparation to give an outline of the nature of the enzyme activity. The finding that this enzyme neither attacks acetylcholine nor is inhibited by high concentration of cholinesterase inhibitors indicates that its nature is basically different from that of cholinesterase. This consideration, together with the observation that this enzyme was inactive toward phosphorylcholine, leads to the conclusion that the choline sulfate hydrolyzing enzyme should be classified as a sulfatase. In fact, the activity of the enzyme was strongly inhibited by sulfite, one of the powerful inhibitors of aryl sulfatase. Examination of the enzyme activity toward various sulfate esters showed, on the other hand, that this enzyme did not appreciably hydrolyze any of the substrates of known sulfatascs. Moreover, the results of inhibition experiments revealed that this enzyme is not inhibited by sulfate, phosphate, fluoride or cyanide, while all types of sulfatascs so far known are reported to be sensitive to one or another of these four anions (16). From these observations it should be concluded that this enzyme represents a new species of sulfatase which specifically acts on choline sulfate, and the author would like to propose the name "choline sulfatase" for the enzyme. Choline sulfatase has a pH optimum around 8.3 and a K_m value estimated to be 4×10^{-2} *M* at pH 8.3 and 37°C.

There is no doubt that choline sulfatase plays an indispensable role in the growth of *Pseudomonas nitroreducens* on choline sulfate, when this sulfate ester is supplied as the sole source of carbon, nitrogen and sulfur. The choline liberated by the action of this enzyme may be used as the source of energy, carbon and nitrogen, and the sulfate may serve as the source of sulfur. Taking into account the adaptive nature of the formation of the enzyme, it is highly probable that the organism does not accumulate choline sulfate when this substance is not added to the growth medium. The metabolic situation of choline sulfate in *Pseudomonas* may thus be

somewhat different from that in the conidiospores of *Aspergillus niger*, in which this sulfate ester is accumulated, as reported by the present author (4). In the latter organism also, choline sulfate was shown to be utilized during the process of germination as the sulfur source for the cellular protein synthesis (17). Comparative studies on the mode of utilization of choline sulfate in *Pseudomonas* and in the conidiospores of *Aspergillus niger* seemed, therefore, to hold considerable promise. However, attempts to detect hydrolysis of choline sulfate by dried or disrupted preparations of germinating spores have so far been unsuccessful under the experimental conditions used for the *Pseudomonas* enzyme. This does not, however, preclude the possibility that choline sulfate in the fungal spores is utilized through hydrolysis by a similar but different sulfatase. Recently, Spencer and Harada (18) made detailed studies of the role of choline sulfate in the sulfur metabolism of fungi, and they suggest that choline sulfate may be utilized through the transfer of sulfate to adenosine-3': 5'-diphosphate to regenerate adenosine-3'-phosphate-5'-phosphosulfate (PAPS). There is, however, no direct evidence for such a mechanism; Kaji and Gregory (5), moreover, have reported that they failed to detect the reversal of choline sulfurylation *in vitro*. The mechanism of choline sulfate utilization in fungal cells, therefore, remains uncovered at present. It may be suggested that there are three alternative possibilities in the utilization of choline sulfate by fungi: a) choline sulfate is hydrolyzed by a choline sulfatase different from that of *Pseudomonas*, b) choline sulfate is utilized through regeneration of PAPS, c) choline sulfate is utilized by some other unknown mechanism.

SUMMARY

A strain of *Pseudomonas* which is able to grow on choline sulfate as its sole organic substrate was isolated from soil. This organism was shown to form adaptively an enzyme catalyzing hydrolysis of choline sulfate into

choline and inorganic sulfate. Studies on the behavior of the partially purified enzyme preparation toward various sulfate and choline esters led to the conclusion that this enzyme is a sulfatase of a so far unknown type, and the name "choline sulfatase" was proposed. Choline sulfatase was shown to have a pH optimum of 8.3 and to be inhibited by sulfite but not by sulfate, phosphate, fluoride or cyanide. The role of this enzyme in choline sulfate metabolism in microorganisms is discussed.

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Studies on Insulin

II. Amino Acid Composition and Terminal Sequences of Bonito Insulin

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It has been well established by Sanger (1) that the genetically determined differences in the chemical structure of mammalian insulins, as far as known, lie in the acidic glycy chain, and only at the special positions of this chain.

As already reported by the present author (2), two different types of insulin were obtained from Langelhans islet of bonito by extraction with acidified and hydrated acetone, fractional precipitations, and further minute chromatographic resolution on a column of carboxymethyl-cellulose. The two insulins so obtained, although having hormonal characters similar to those of mammalian insulins, differed from each other in their

N-terminal residues. The bonito insulin-I had one glycy and one leucyl chains, and bonito insulin-II had one glycy and one alanyl chains, as compared with one glycy and one phenylalanyl chains in mammalian insulins. These facts suggested that the structural differences between mammalian and fish insulins also occurred in their basic chains.

It is suggested in this paper that bonito insulin-II seems to possess the above partial structure.

EXPERIMENTAL

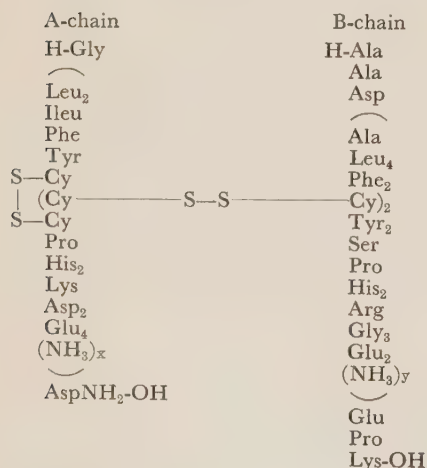
Materials—Bonito insulin-II used was obtained by the same procedures as described in the previous paper (2) and was further purified by rechromatography. Recent studies in this laboratory indicated that both bonito insulins -I and -II, previously obtained by gradient elution chromatography (2), were not completely homogeneous and two other minor components were further resolved by rechromatography with smaller concentration gradient. This new preparation II, however, was almost free from other components (less than three per cent).

Carboxypeptidase-A used for C-terminal analysis, was recrystallized six times and treated with diisopropylphosphoryl fluoride, according to the usual method (4).

The oxidized glycy and alanyl chains of bonito insulin-II was resolved, after oxidative cleavage of inter-peptide disulfide linkages with performic acid (5), by fractional precipitation of the oxidized alanyl chain with *N* trichloroacetic acid. In a typical example, 50 mg. of insulin was dissolved in 2 ml. of 90% formic acid and 0.2 ml. of 30% hydrogen peroxide was added. The mixture, after standing at room temperature for 30 minutes, was diluted with 10.3 ml. of water and mixed with 12.5 ml. of 2.0 *N* trichloroacetic acid. The precipitate collected by centrifugation was washed

SCHEME I

Partial Structure of Bonito Insulin-II*



* Abbreviations for the amino acid residues are those proposed by E. Brand (3).

once with *N* trichloroacetic acid and several times with ether. N-Terminal analysis by the usual 1-fluoro-2,4-dinitrobenzene method indicated the precipitate fraction to be the oxidized alanyl chain (purity, 96 per cent; yield, 30 mg.).

The supernatant of *N* trichloroacetic acid-precipitation mentioned above, was continuously extracted with ether to remove the acid. The resulting aqueous layer was concentrated at 40°C and a large volume of a mixture of acetone and ether (3:1, v/v) was added. The resulting precipitate was the oxidized glycyl chain (purity, 98%; yield, 20 mg.) (6).

Amino Acid Composition—Acid hydrolysate of a sample to be analyzed was obtained by heating 5 to 10 mg. of protein in a sealed glass tube with 6 *N* hydrochloric acid (0.5 ml.) at 110°C for 24 hours and by repeated evaporation of the resulting hydrolysate over sodium hydroxide in an evacuated vessel. The amino acid composition of the residue was determined by the following two different methods.

Method A: A part of acid hydrolysate was dinitrophenylated with 1-fluoro-2,4-dinitrobenzene and the dinitrophenylated amino acids were resolved by two-dimensional paper chromatography. The amino acid content was calculated from the optical density of the extract from each yellow spot. Molar extinction coefficients reported by Rao *et al.* (7) were used for these calculations. No correction for the recovery from paper was made.

Method B: Another part of the acid hydrolysate was directly resolved on a paper sheet by two-dimensional chromatography, using 1-butanol-acetic acid-water (4:1:2, v/v) and phenol-water (4:1, v/v) mixtures as the solvent systems. Each spot of amino acid was detected and assayed by trinitrophenylation with 2,4,6-trinitrobenzene-1-sulfonic acid at pH 8. The details of this procedure have already been published by Satake (8).

The absence of free sulfhydryl group and tryptophanyl residue in intact bonito insulin (before performic acid-oxidation) was proved by the method of Jansen (9) and from the ultraviolet absorption spectrum, respectively.

N-Terminal Structure—The usual 1-fluoro-2,4-dinitrobenzene method was used. For the analysis of N-terminal residues, dinitrophenylated sample was hydrolyzed with 6 *N* hydrochloric acid at 110°C for 5 hours (2) and partial hydrolysis was achieved with 12 *N* hydrochloric acid at 37°C for 3 days.

These hydrolysates were extracted with ether (thrice) and then with ethyl acetate (thrice), and the dinitrophenyl derivatives still remaining in the aqueous layer were separated from other components by the

use of talc-column (10). The dinitrophenyl derivatives soluble in ether (dinitrophenol being eliminated by sublimation), ethyl acetate and water so fractionated were separately resolved by paper chromatography with the use of 1-butanol saturated with 5% ammonia and 1.5 *M* sodium phosphate buffer (pH 6) as the solvent systems.

C-Terminal Structure—The C-terminal structure was examined by the use of carboxypeptidase-A, according to the method of Neurath (11). The amino acids liberated by the action of this enzyme were identified and assayed as their dinitrophenyl derivatives (12).

The presence of lysine at the C-terminal of alanyl chain was verified by the use of thiohydantoin method, according to the method of Waley and Watson (13). The thiohydantoin derived from the C-terminal group with a mixture of ammonium thiocyanate and acetic anhydride was extracted with ethyl acetate and was identified as the corresponding amino acid after being regenerated by hydrogen bromide at 155°C.

RESULTS

Isolation of the Alanyl and Glycyl Chains from Bonito Insulin-II Oxidized with Performic Acid—

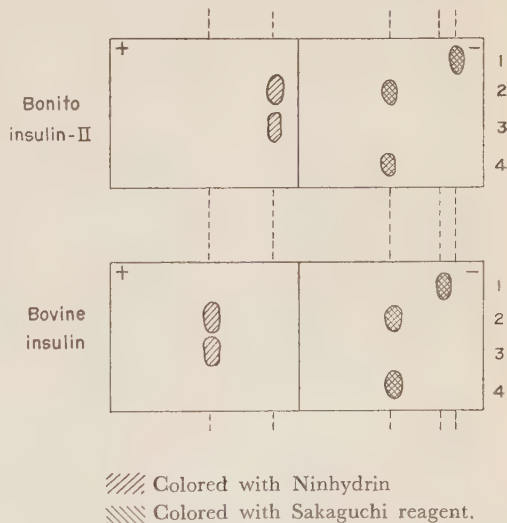


FIG. 1. Paper electrophoretic patterns of intact and oxidized insulins.

1) Intact insulin. 2) Performic acid-treated insulin. 3) *N* Trichloroacetic acid-soluble fraction derived from 2). 4) *N* Trichloroacetic acid-insoluble fraction derived from 2). Conditions were the same as those described in the preceding paper (2).

Fig. 1 shows the paper electrophoretic patterns of intact and performic acid oxidized-insulins. The electrophoretic resolution of insulins into two components, only after the performic acid-treatment, seemed to indicate that bonito insulin-II as well as mammalian ones (I) possessed inter-peptide disulfide linkage(s) between the two different chains. In the preceding paper (2), the more acidic component (glycyl chain) of oxidized bonito insulin-II was described as Sakaguchi reaction-positive. Soon after the publication, however, this was found to be due to the contaminated glycyl chain of insulin-IIb which contained arginine instead of lysine in insulin-II*.

* Kotaki, A., *et al.*, unpublished data.

The preparative isolation of the two chains cleaved by oxidation, was achieved by the fractional precipitation of less acidic component (alanyl chain) with *N* trichloroacetic acid, according to the procedure described in the experimental part. Results that were reproducible and almost quantitative were readily obtained, either on fish or on mammalian oxidized insulin (Fig. 1) (see also (6)). According to the literature (14), such a fractional precipitation of oxidized mammalian insulins had been performed with the use of acetic acid and ammonia. By this method, however, the yields of both chains were far from quantitative, especially in the case of bonito insulin-II.

TABLE I

Amino Acid Composition of Oxidized Bonito Insulin-II, Expressed as the Number of Amino Acid Residues

Amino acids	Alanyl chain			Glycyl chain		
	Number of residues per mole (3,500 g.)			Number of residues per mole (2,500 g)		
	DNP-method ^{a)}	TNP-method ^{b)}	Number to ^{d)} nearest integer	DNP-method ^{a)}	TNP-method ^{b)}	Numbers to ^{d)} nearest integer
Asp	} 4.57(±2.7%)	1.02(±0.9%)	1 (1)	} 6.79(±2.7%)	2.73(±6.1%)	3 (2)+1 ^{e)}
Glu		2.97(±4.4)	3 (3)		4.09(±5.5)	4 (4)
Ser	0.97(±2.1)	1.11(±8.8)	1 (1)	none	none	0 (2)-2
Gly	2.88(±3.5)	3.05(±2.5)	3 (3)	1.02(±2.1)	1.14(±2.6)	1 (1)
Thr	none	none	0 (1)-1 ^{e)}	none	none	0 (0)
Tyr	c)	1.66(±1.6)	2 (2)	c)	0.74(±2.3)	1 (2)-1
Pro	2.20(±0.4)	c)	2 (1)+1	1.00(±9.5)	c)	1 (0)+1
Phe	1.87(±2.1)	2.02(±5.6)	2 (3)-1	1.01(±4.3)	1.10(±5.7)	1 (0)+1
Ala	2.89(±1.9)	2.87(±2.7)	3 (2)+1	none	none	0 (1)-1
Val	2.10(±2.7)	2.00(±1.3)	2 (3)-1	none	none	0 (2)-2
Leu	} 4.17(±1.9)	3.98(±5.3) ^{f)}	4 (4)	} 3.14(±3.1)	3.06(±4.7) ^{f)}	2 (2)
Ileu			0 (0)			1 (1)
Lys	c)	1.03(±2.7)	1 (1)	c)	1.17(±3.6)	1 (0)+1
Arg	c)	0.98(±8.2)	1 (1)	c)	none	0 (0)
His	c)	2.13(±7.2)	2 (2)	c)	1.80(±7.1)	2 (0)+2
CySO ₃ H	c)	2.13(±1.2)	2 (2)	c)	4.40(±4.1)	4 (4)
MetSO ₂	none	none	0 (0)	none	none	0 (0)
Total			29 (30)-1			21 (21)±0

a) Method A. b) Method B. All figures are the means of three determinations, whose deviations are also shown as their percentage in parentheses. c) Not determined. d) The figures in parentheses are the values of corresponding chain in bovine insulin. e) Difference between bonito and bovine insulins. f) Quantitative differentiation of leucine and isoleucine was achieved by multi-development method (see Fig. 2).

Amino Acid Compositions of the Oxidized Alanyl and Glycyl Chains—As the preliminary experiments on bonito insulin-II (before treatment with performic acid) indicated the absence of tryptophan, cysteine, and cysteic acid residues, the amino acid composition was analyzed only on the acid hydrolysate of each performic acid-oxidized chain, and all of cysteic acid found was assumed to be derived from cystine residues. For the assay of each amino acid content in acid hydrolysate, two different methods were used, dinitrophenylation (DNP) and trinitrophenylation (TNP) methods. The results that were reproducible to ± 5 per cent, were readily obtained by both methods.

These results, as summarized in Table I, were different from those reported on mammalian insulin-A and -B. As regards bovine insulin-A (glycyl chain), the glycyl chain of bonito insulin-II was likewise constructed from 21 amino acid residues, including 4 of half cystine residues. It contained, however, no serine (two less), alanine (one less), or valine (two less), all of which were present in bovine insulin-A, but did contain one proline, one lysine, two histidine, and one phenylalanine, all of which were absent in bovine insulin-A. It possessed, moreover, one less tyrosine (only one) and one more aspartic acid (three) residues. Thus, the glycyl chain of bonito insulin-II differed from bovine insulin-A in six amino acid residues.

The identification of leucine and isoleucine was accomplished by one-dimensional

paper chromatography multi-developed with a freshly prepared mixture of 1-butanol, acetic acid, and water (4:1:5, v/v) according to the method of Block (15). The result indicated that bonito insulin-II contained one isoleucine in the glycyl chain (A), just like that of bovine insulin (Fig. 2).

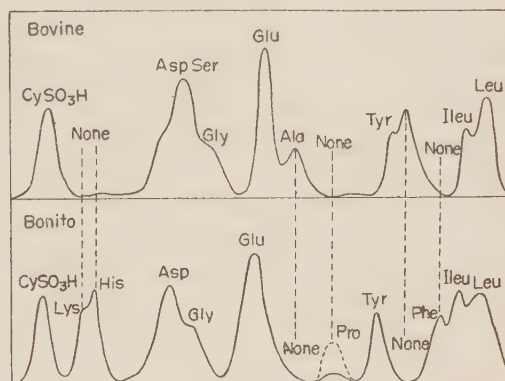


FIG. 2. Amino acid constituents of the glycyl chains of bovine and bonito insulins.

Acid hydrolysate was resolved by multi-development technique and the ninhydrin-colored spots were measured by densitometer at 570 and 485 $m\mu$ (only for proline).

The amino acid composition of alanyl chain, on the other hand, was fairly similar to that of mammalian insulin-B (Phenylalanyl chain). The alanyl chain in bonito insulin-II contained only one more proline (two) and alanine (three), and only one less threonine

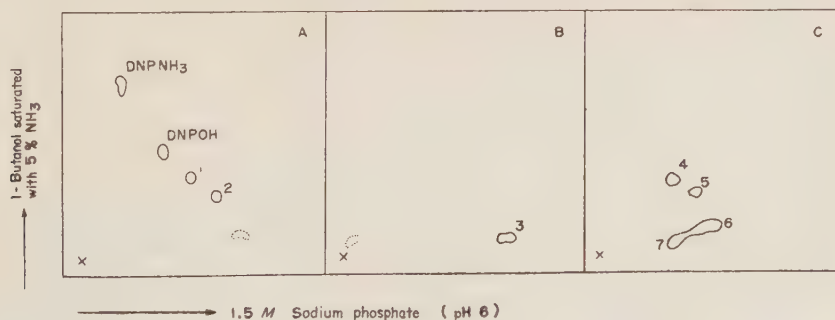


FIG. 3. Paper chromatograms of dinitrophenyl compounds derived from dinitrophenylalanyl chain with acid.

(A) Ether-soluble; (B) Ethyl acetate-soluble; (C) Water-soluble.

(one), valine (two), and phenylalanine (two) residues than mammalian insulin-B. It should be noted, however, that the alanyl chain of bonito insulin-II was constructed from 29 (one less) amino acid residues.

N-Terminal Structure—Partial acid hydrolyzate of dinitrophenylalanyl chain (12 *N* hydrochloric acid, at 37°C for three days) was fractionated into ether-, ethyl acetate- and water-soluble parts, and then dinitrophenyl compounds in each fraction were resolved by paper chromatography. Fig. 3 illustrates their paper chromatograms. The main spots on paper were separately eluted, and their amino acid compositions were determined after desalting and acid hydrolysis.

The results hereby obtained, as summarized in Table II, revealed that the N-terminal H-Phe-Val-AspNH₂—residue of mammalian insulin-B seemed to be substituted by H-Ala-Ala-Asp (or AspNH₂)—in bonito insulin-II. Such a substitution will give a reasonable explanation why the alanyl chain contained one less phenylalanine and valine, and one (not two*) more alanine residues than those of mammalian insulin-B.

TABLE II

*Amino Acid Composition of Dinitrophenyl Compounds
Obtained from Partial Hydrolysis of
Dinitrophenylalanyl Chain*

Spot No.	Amino acid composition	Presumed structure
1	DNP-Ala	H-Ala
2	DNP-Ala, Ala	H-Ala-Ala
3	DNP-Ala, Ala, Asp	H-Ala-Ala-Asp
4	ϵ -mono-DNP-Lys	Lys-OH
5	ϵ -mono-DNP-Lys, Pro	Pro-Lys-OH
6	ϵ -mono-DNP-Lys, Pro, Glu	Glu-Pro-Lys-OH
7	DNP-Ala, Ala, Asp, Pro, Leu CySO ₃ H, dinitroaniline, etc.	?

Under similar conditions, the glycyl chain of bonito insulin-II gave dinitrophenylglycine as the sole main component in the ether-

* The alanyl chain had 29 amino acid residues ending in—Pro-Lys-OH, while the phenylalanyl chain had 30, ending in—Pro-Lys-Ala-OH (see also the section on “C-terminal Structure”) (24).

soluble fraction (dinitrophenol being removed by sublimation) and the amount of ethyl acetate-soluble dinitrophenyl compounds was only a trace. The remaining aqueous layer, after extractions with both solvents, still contained dinitrophenylglycyl peptids, together with ϵ -monodinitrophenyl-lysine and its peptides. However, the fraction was a mixture too complicated to be resolved into pure components.

C-Terminal Structure—Fig. 4 shows the amino acids liberated from insulins oxidized with performic acid by the action of carboxypeptidase-A. Glycyl chains of bonito and bovine insulins liberated asparagine and a minute amount of aspartic acid in a similar way, but the alanyl chain was resistant against the peptidase, differing from mammalian insulin-B which liberated alanine rapidly. These results seemed to indicate the presence of genetically determined difference between bonito and bovine insulin-B, at their C-terminals, as well as at their N-terminals.

These results were further verified by the thiohydantoin method of Waley and Watson (13). Thus, the qualitative analysis re-

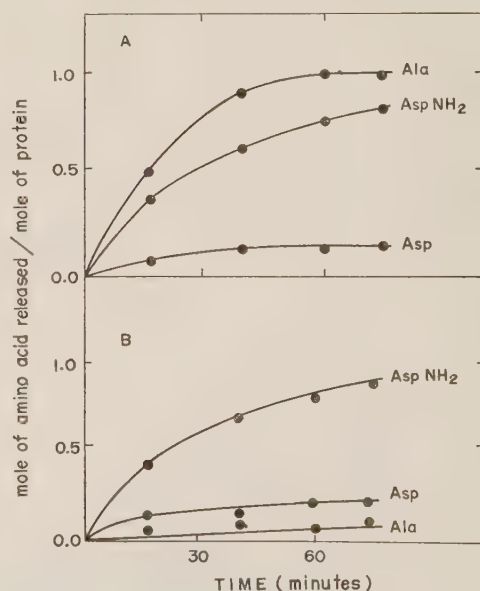


FIG. 4. Amino acids liberated from oxidized insulins by the action of carboxypeptidase A.

(A) Oxidized bovine insulin. (B) Oxidized bonito insulin-II.

vealed the presences of asparagine* at the C-terminal of glycyl chain and of lysine at that of alanyl chain.

As already described in this paper, the alanyl chain of bonito insulin-II possessed only one lysine residue (see Table I) and its partial hydrolysis gave two kinds of lysine-containing peptides, (Pro, Lys) and (Glu, Pro, Lys) (see Table II). These results seemed to indicate that the C-terminal structure of—Thr-Pro-Lys-Ala-OH in bovine insulin-B was substituted with—Glu (or GluNH₂)-Pro-Lys-OH in the alanyl chain of bonito insulin-II. Such a substitution will give a reasonable explanation why the latter chain contains no threonine and one less amino acid residues (24) as compared with bovine insulin-B.

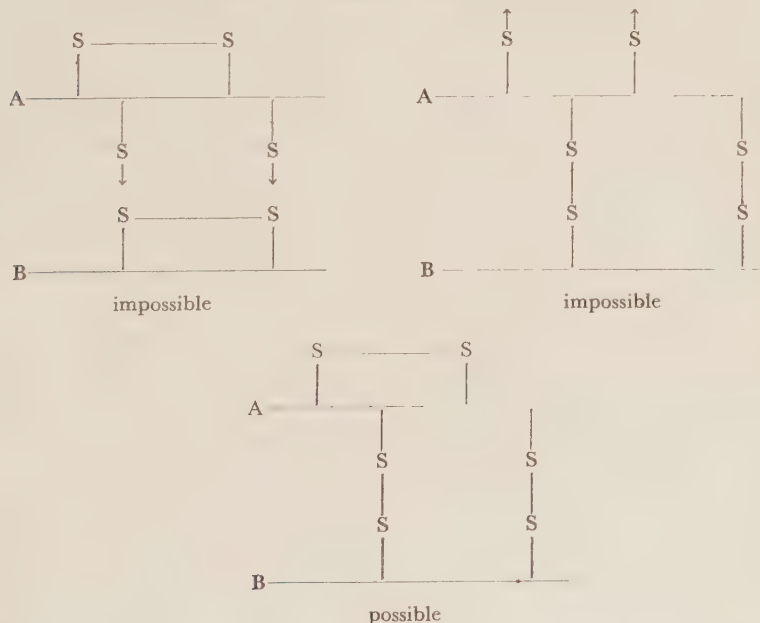
DISCUSSION

The above-mentioned results will suggest

the partial structure of bonito insulin-II to be as shown in Scheme I. Intact bonito insulin-II, as well as mammalian ones, had neither cysteine nor cysteic acid residue, while the oxidized glycyl chain possessed four cysteic acid residues and the oxidized alanyl chain, two (Table I). These results indicate the presence of three disulfide linkages in bonito insulin-II, either as inter- or intra-peptide linkages. The presence of former linkage(s) was presumed from the electrophoretic resolution of bonito insulin-II, only after the performic acid-treatment (Fig. 1). Assuming the molecular weight of this protein to be 6×10^3 (2), these results seemed to suggest the presences of two inter-peptide disulfide linkages between the glycyl and alanyl chains, and one intra-peptide disulfide linkage in the glycyl chain, just the same as in mammalian insulins (Scheme I) (16).

SCHEME II

*Possible Structure of Disulfide Linkages in Bonito Insulin-II***



* Identified as aspartic acid.

** The molecular weight of this protein was assumed to be 6×10^3 .

The preparative fractionation of A and B chains had been achieved both by (i) oxidative cleavage of the disulfide linkages with performic acid, followed by fractional precipitation (14), counter current distribution (17),

chromatography (18), or electrophoresis (19), and by (ii) reductive cleavage of the interpeptide disulfide linkages by protecting the reactive sulfhydryl groups and then by electrophoresis (20) or chromatography (21). The fractional isolation of oxidized insulins with *N* trichloroacetic acid used in the present work seemed to be better than those methods mentioned above in its quantitative yield and its simplicity, either in preparative or analytical scale. It was of interest that S-carboxymethylated or S-sulfonated insulin-A and -B were also fractionated from each other with trichloroacetic acid*.

The amino acid composition of bonito insulin-II was different from those reported on mammalian ones (1), both in the A- and B-chains. The structural difference in the B-chains should be emphasized here; as the genetical difference in the chemical structure of insulins, as far as mammalian ones were concerned, was in the A-chain and only at the 8th, 9th, and 10th amino acid residues from the N-terminal.

From the studies on proteinase-modified bovine insulin (22), it had been presumed that the C-terminal alanine in the B-chain, differing from the C-terminal asparagine in the A-chain, was not essential for the hormonal activity, while the several amino acid residues preceding the C-terminal alanine were essential. The substitution of -Thr-Pro-Lys-Ala-OH (in mammalian insulin) with Glu (or GluNH₂)-Pro-Lys-OH (in fish insulin)** is interesting, and the substitution of N-terminal H-Phe-Val-AspNH₂- residue with H-Ala-Ala-Asp (or AspNH₂-) at the B-chain is also of interest, because leucine aminopeptidase-modified bovine insulin was reported still possess the hormonal activity, thus indicating that several amino acid residues from the N-terminal were not essential to the hormonal

activity (23). Except for the fairly different terminal structures noted above, bovine and bonito insulin-B have rather similar compositions. This fact may suggest the location of genetically determined differences only at the terminal parts and suggest the presence of a common or a similar structure throughout the wide ranges of their polypeptide chains (between 4th and 26th amino acid residues from their N-terminals*).

Both A-chains of bovine and bonito insulins had the same N- (glycine) and C- (asparagine) terminal residues, but showed remarkable difference in their compositions. As already reported in the preceding paper (2), two glycyll chains could be distinguished from each other by their behavior in electrophoresis and counter current distribution (see also Fig. 1). Such a remarkable difference seemed not to be surprising, as their amino acid composition differed as much as 30 per cent (6 out of 21 residues). The glycyll chain of bonito insulin-II contained one proline, one phenylalanine, and three basic amino acids, all of which were absent in bovine insulin-A, but did not contain, serine, alanine, or valine, all of which were found in bovine insulin-A. Such a composition also indicated that both glycyll chains are able to possess a similar structure only in their latter half of polypeptide chain from N-terminals (between 14th and 21st amino acid residues).

SUMMARY

1. Bonito insulin-II contained two different polypeptide chains, glycyll (A) and alanyl (B), which could be resolved only after treatment with performic acid.

2. Electrophoretic and amino acid analyses suggested the presence of three disulfide linkages, two as interpeptide linkage and one as intra-peptide linkage in the A-chain.

3. Amino acid compositions of alanyl (bonito) and phenylalanyl (bovine) chains were fairly similar, although the sequences of ala-

* Kotaki, A., *et al.*, Unpublished data.

** S. Akabori, and H. Ohno had already found with the use of hydrazinolysis technique that bonito insulin (presumably a mixture of insulin-I and -II) had -Pro-LysOH as the C-terminal, differing from mammalian ones which had -Pro-Lys-Ala-OH (24).

* In these ranges, the bonito insulin-II possessed only one more proline and one less glutamic acid (or glutamine) than mammalian insulin.

nyl chain was H-Ala-Ala-Asp (or AspNH₂)—Glu (or GluNH₂)—Pro-Lys-OH instead of H-Phe-Val-AspNH₂—Thr-Pro-Lys-Ala-OH in the mammalian insulins.

4. Amino acid composition of the A-chain differed as much as 30 per cent (=6/21) from the corresponding bovine one although they had just the same N- and C-terminals.

The author wishes to express his thanks to Prof. K. Satake for his valuable guidance and encouragement during the course of this work.

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Studies on Laccases of Lacquer Trees

I. Comparison of Laccases Obtained from *Rhus vernicifera* and *Rhus succedanea*

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The occurrence of the enzyme laccase in the latex of Japanese lacquer tree (*Rhus vernicifera*) was first discovered in 1883 by Yoshida (1), and about ten years later the enzyme was partially purified by G. Bertrand (2, 3). Since then, laccases occurring in latexes of the other species of lacquer trees have also been studied. Thus, the enzyme of Indo-Chinese lacquer tree (*Rhus succedanea*) has been the subject of investigation by Keilin and Mann (4, 5), Yakushiji (6), D. Bertrand (7-12), and Tissières (13, 14), and that of Burmese lacquer tree (*Melanorrhoea usitata*) by Keilin and Mann (5). More recently, Nakamura (15) has purified the laccase of Chinese lacquer tree (*Rhus vernicifera*) by means of ion-exchange chromatography and reported its physico-chemical properties. However, the data reported by these workers are sometimes incompatible with each other and it seems very important to reinvestigate the properties of laccases with preparations sufficiently purified from well-defined latexes of lacquer trees.

In the present investigation laccases of Japanese and Indo-Chinese lacquer trees have been purified to a satisfactory degree by column chromatography on an ion-exchange resin, and their properties have been compared. A preliminary account of this work based on the results obtained with partially purified enzyme preparations has been reported elsewhere (16).

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EXPERIMENTALS

Purification of R. vernicifera Laccase—Crude latex of *R. vernicifera* collected in Ibaraki Ken was used as the starting material. The latex had been stored below 5°C for one to six months before use.

Three liters of acetone were added to 1 kg. of the latex at room temperature with stirring, and the precipitate collected by filtration was washed with acetone. The addition of acetone to the latex at room temperature caused no loss in laccase activity, although purified laccase was found to be partly inactivated by acetone at room temperature. The acetone-washed precipitate was then extracted with 500 ml. of water, and the insoluble materials were removed by filtration. The crude extract thus obtained was saturated with ammonium sulfate at 25°C, and the precipitate containing laccase was collected by centrifugation and dissolved in 150 ml. of water. The solution was made 65% saturated with respect to ammonium sulfate, and the brown precipitate formed was centrifuged off and discarded. The supernatant was then saturated with ammonium sulfate and the laccase-containing precipitate was collected by centrifugation and dissolved in 50 ml. of 0.01 M phosphate buffer of pH 6.5. The enzyme was again precipitated from the solution by the addition of 0.8 volume of cold acetone at 0°C, and the blue precipitate was collected by centrifugation and dissolved in 10 ml. of 0.05 M sodium phosphate buffer of pH 5.5.

The solution was then applied to a 4×50 cm. column of Amberlite XE-64 which had been equilibrated with 0.05 M sodium phosphate buffer of pH 5.5, and the column was eluted for 15 to 20 hours with the same buffer. At the end of elution, three distinct colored bands were formed on the column. A strongly blue-colored band remaining at the top of the column and a narrow yellow band formed

just below the former were found to contain no laccase activity. All of the laccase activity was found to be associated with a broad blue band which had migrated farthest on the column. The resin bearing this blue band was cut off from the column and eluted with cold $0.1 M Na_2HPO_4$. Laccase was then precipitated from the eluate with acetone, dissolved in distilled water, and dialyzed against distilled water. About 200 mg. of purified *R. vernicifera* laccase was thus obtained from 1 kg. of the crude latex. A summary of a typical purification experiment is recorded in Table I.

The solution of the purified enzyme was very stable and could be stored for several weeks in a refrigerator without any loss of activity.

The strongly blue-colored band remaining at the top of the column was also eluted with $0.2 M Na_2HPO_4$. The eluate was found to contain a blue protein lacking laccase activity. Further purification and properties of this new blue protein will be reported in a separate paper of this series.

Purification of *R. succedanea* Laccase—Crude latex of *R. succedanea* imported from Phu Tho Province, North Viet Nam, was used as the starting material.

The enzyme was precipitated with acetone and extracted with water just as in the case of *R. vernicifera* laccase. The crude extract was saturated with ammonium sulfate, and the precipitate formed was collected and dissolved in water. The solution was then fractionated with ammonium sulfate and the fraction precipitating between 60 and 80% saturation was collected and dissolved in $0.01 M$ phosphate buffer of pH 6.5. The laccase was again precipitated from the solution by the addition of 0.8 volume of

cold acetone at $0^\circ C$, and the green precipitate was dissolved in $0.05 M$ sodium phosphate buffer of pH 5.5.

The solution was then applied to a column of Amberlite XE-64 equilibrated with $0.05 M$ sodium phosphate buffer of pH 5.5 and eluted with the same buffer. The affinity of this laccase to the resin was much less than that of *R. vernicifera* laccase under these conditions. The laccase was therefore scarcely adsorbed on the resin and passed through the column rather rapidly followed by yellow impurities. A blue component devoid of laccase activity and brown impurities, which had not been removed by ammonium sulfate fractionation and acetone precipitation, remained adsorbed on the upper part of the resin column.

The blue effluents containing laccase activity were combined and the laccase was recovered by acetone precipitation and dissolved in $0.05 M$ sodium phosphate buffer of pH 5.5. The solution was again subjected to column chromatography on Amberlite XE-64 as before and the laccase was again precipitated from the blue effluents and dissolved in water. The solution containing purified enzyme was finally dialyzed against distilled water. About 50 mg. of purified laccase was obtained from 1 kg. of crude latex. Table I also contains a summary of this purification.

Activity Measurement—The activity of laccase preparations was determined by the conventional manometric technique. The rate of oxygen uptake measured was expressed in terms of the quotient Q_{O_2} , i. e. $\mu l. O_2/mg.$ dry weight/hour. The standard reaction mixture consisted of 1 ml. of enzyme solu-

TABLE I

Purification of the Laccases

Activity recovery and specific activity at several purification steps.

Purification Steps	<i>R. vernicifera</i> laccase		<i>R. succedanea</i> laccase	
	Specific activity $Q_{O_2}^{1)}$	Activity recovery %	Specific activity $Q_{O_2}^{1)}$	Activity recovery %
Crude latex	2.0×10	—	6.8×10	—
Crude extract	4.1×10^2	100	7.0×10^2	100
Before resin treatment	3.8×10^3	80	6.1×10^3	65
Final preparation	8.5×10^3	25	7.4×10^4	28

1) Activity of the enzymes was assayed at $25^\circ C$ in the presence of $0.033 M$ hydroquinone.

tion, 1 ml. of 0.13 *M* phosphate buffer, and 1 ml. of 0.1 *M* substrate. Gas phase was air and the reaction was carried out at 25°C. As will be shown later, optimum pH for *R. vernicifera* laccase is higher than that for *R. succedanea* enzyme. The activities of the two enzymes were therefore usually measured at different pH's; pH 6.5 for *R. succedanea* laccase and pH 7.0 for *R. vernicifera* laccase. pH 6.5 is the optimum condition for *R. succedanea* laccase activity. The optimum pH for *R. vernicifera* laccase is 7.5, but at higher pH's than 7.0 the autoxidation of the substrate becomes appreciable, and it was inadequate to carry out routine activity measurements under such condition.

Detection and Determination of Copper—Metal constituents of the laccase preparations were detected by emission spectrography and by X-ray fluorescence spectroscopic analysis.

About 200 mg. of laccase was dried at 110°C in a fused-silica crucible and ashed by igniting over flame. The ash was well mixed with 40 mg. of pure sodium chloride by grinding in an agate mortar, and the mixture was tamped into a hole in a carbon electrode. Emission spectrography using the cathode-layer d. c. arc was carried out in a Shimadzu model QL-170 quartz spectrograph. For X-ray fluorescence spectroscopic analysis, the ash was pasted on a small strip of filter paper with an acetone solution of

polyvinyl acetate. The paper strip was then examined by a Rigaku Denki 'Geigerflex' automatic recording X-ray fluorescence spectrometer.

Quantitative analysis of copper was performed by the dithione method using carbon tetrachloride as the solvent. The sample was wet-ashed by heating with 2 ml. of concentrated sulfuric acid.

RESULTS

Criteria of Purity—Fig. 1 is a reproduction of sedimentation patterns of purified *R. vernicifera* and *R. succedanea* laccases as obtained in a Spinco model E analytical ultracentrifuge. As can be seen, both of the laccase preparations showed only one symmetrical peak indicating their ultracentrifugal homogeneity. Molecular weight determinations based on their sedimentation and diffusion behaviors will be described in a later section of this paper.

Electrophoretic characteristics of the two laccase preparations were also studied by using a Hitachi model HTB Tiselius type apparatus. As will be seen from Fig. 2, the purified preparation of *R. vernicifera* laccase was also found to be homogeneous electro-

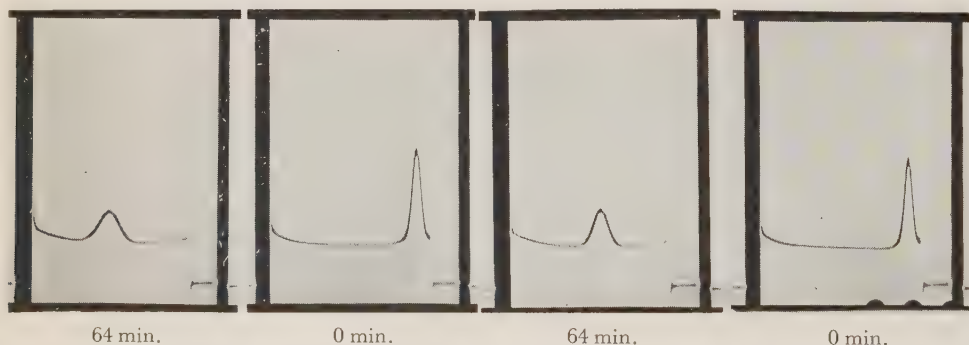


FIG. 1. Sedimentation patterns of *R. vernicifera* laccase (right) and *R. succedanea* laccase (left) in 0.1 *M* phosphate buffer of pH 6.5. Protein concentrations, 0.42 g./100 ml. (right), and 0.59 g./100 ml. (left). Rotor speed, 59,780 r.p.m. Rotor temperature, 11.0°C (right) and 14.3°C (left).

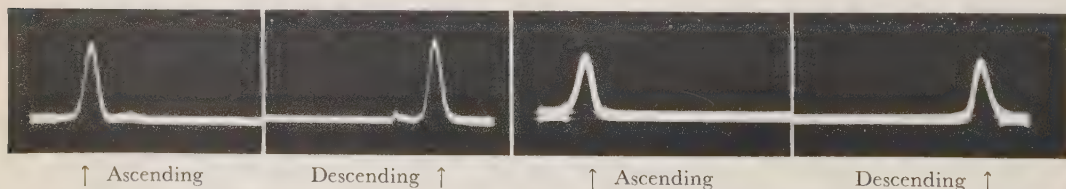


FIG. 2. Electrophoretic patterns of *R. vernicifera* laccase (right) and *R. succedanea* laccase (left) in 0.05 *M* phosphate buffer of pH 7.0. Protein concentrations, 0.9 g./100 ml. (right) and 1.4 g./100 ml. (left). Current, 8.0 mA using a cell with a section area of 0.3 cm². Temperature was 17–18°C. Time of electrophoresis was 160 min. Position of initial boundary is indicated by the arrows.

phoretically. The electrophoretic pattern of the purified enzyme obtained from *R. succedanea*, however, revealed the presence of a very small amount of impurities which moved faster than the main component.

Catalytic Activity—Both of the laccase preparations were found to catalyze the aerobic oxidation of hydroquinone, catechol, *p*-phenylenediamine, and L-ascorbic acid. The oxidation of hydroquinone and L-ascorbic acid catalyzed by the two preparations proceeded linearly for more than one hour. As shown in Fig. 3, however, when catechol was used as the substrate, *R. vernicifera* laccase was found to be progressively inactivated during the reaction. *R. succedanea* laccase, on the other hand, showed slight reaction-inactivation even when catechol was used as the substrate.

The enzyme from *R. succedanea* was very highly active; Q_{O_2} of the purified preparation being $7.5\text{--}8.5 \times 10^4$ in the oxidation of hydroquinone. The activity of *R. vernicifera* laccase was much less than that of *R. succedanea* enzyme. As is shown in Table II, Q_{O_2} of *R. vernicifera* laccase was about one tenth of that of *R. succedanea* enzyme. K_m values for hydroquinone and catechol recorded in Table II are also different in the two laccases.

TABLE II

Q_{O_2} , Optimum pH, and K_m of *R. vernicifera* Laccase and *R. succedanea* Laccase

	<i>R. vernicifera</i> laccase	<i>R. succedanea</i> laccase
Q_{O_2} : Hydroquinone	$8.0\text{--}9.0 \times 10^3$	$7.5\text{--}8.5 \times 10^4$
Optimum pH:		
Hydroquinone	7.5	6.4
// : Catechol	7.6	6.6
// : L-Ascorbate	7.6	6.6
K_m : Hydroquinone	$3 \times 10^{-1} M$	$1.9 \times 10^{-2} M$
// : Catechol	$4.5 \times 10^{-2} //$	$1.5 \times 10^{-2} //$

Especially, a very high K_m value of *R. vernicifera* enzyme for hydroquinone is quite noticeable.

The effect of hydrogen ion concentration on the activity of the two laccases is shown

in Fig. 4 and Table II. It will be seen that *R. succedanea* enzyme has its optimum pH at a considerably more acid region as compared with that of *R. vernicifera* enzyme. The pH-activity curves of crude extracts in the oxidation of both hydroquinone and catechol were almost identical with those of the purified preparations.

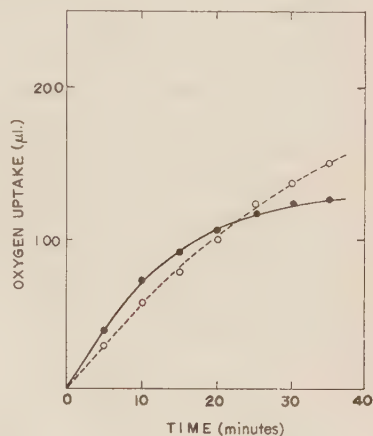


FIG. 3. Oxidation of catechol by *R. vernicifera* laccase and *R. succedanea* laccase. Conditions as described in Experimental.

— *R. vernicifera* laccase
 ---- *R. succedanea* laccase

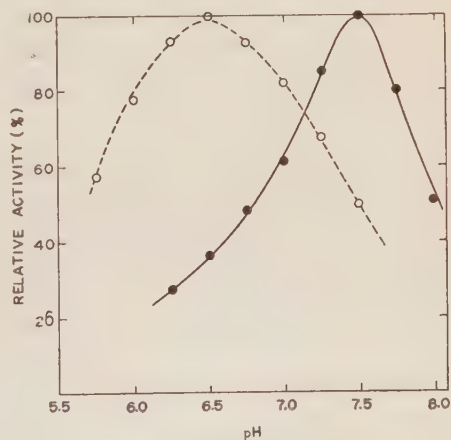


FIG. 4. pH-Activity curves of the two laccases in the oxidation of hydroquinone.

— *R. vernicifera* laccase
 ---- *R. succedanea* laccase

Inhibitors and Heat Stability—In the present study it was confirmed that both laccases are

inhibited by Na_2S , KCN, and NaN_3 as already reported by previous workers (4). Carbon monoxide, ethylenediamine tetraacetate, and thiourea exhibited no inhibitory actions.

Although the two enzymes were found to be irreversibly inactivated concomitant with the disappearance of their blue color when heated in solutions, it was revealed that there is a significant difference in their heat stability. The results illustrated in Fig. 5 show that *R. succedanea* laccase is more resistant to heat than *R. vernicifera* enzyme; the former is almost stable when heated to 50°C , while the latter is rapidly inactivated at the same temperature. The crude extracts from latexes of the two lacquer trees also showed similar heat stability properties.

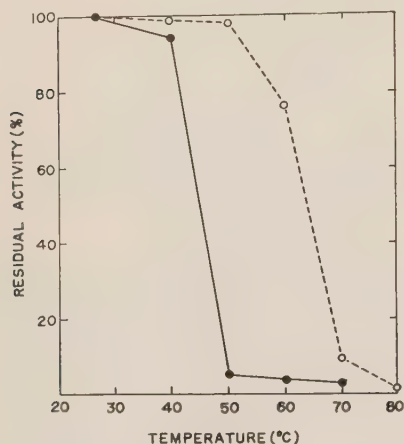


FIG. 5. Heat inactivation of the laccases in dilute solutions. Solutions of the laccases (0.5–2.0 mg./ml.) in 0.13 M phosphate buffer of pH 6.5 were kept at various temperatures for 30 minutes. After heat treatments, residual activity was determined at 25°C .

— *R. vernicifera* laccase
 ---- *R. succedanea* laccase

Absorption Spectra—Figs. 6 and 7 show the absorption spectra of *R. vernicifera* and *R. succedanea* laccases, respectively, measured by a Hitachi model UCA-1 spectrophotometer. *R. vernicifera* enzyme shows two absorption maxima at $280\text{ m}\mu$ and $617\text{ m}\mu$; *R. succedanea* enzyme also shows two absorption maxima at $280\text{ m}\mu$ and $609\text{ m}\mu$. The spectra of both enzymes have a shoulder at about $335\text{ m}\mu$.

The absorption peaks in the red region disappeared on reduction.

Since the absorption in the red region is believed to be due to the presence of copper in the enzyme molecule, molar extinction coefficients of the enzymes per atom of copper were calculated at their absorption maxima in the red region. The values obtained were $1.24 \times 10^3/\text{cm.}/\text{atom Cu/liter}$ for *R. vernicifera* laccase at $617\text{ m}\mu$ and $1.15 \times 10^3/\text{cm.}/\text{atom Cu/liter}$ for *R. succedanea* laccase at $609\text{ m}\mu$.

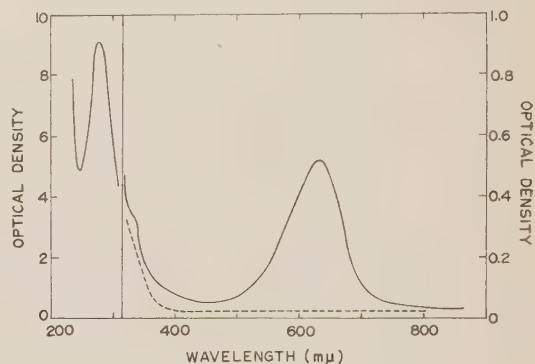


FIG. 6. Absorption spectra of *R. vernicifera* laccase. 1.00% solution in 0.13 M phosphate buffer of pH 7.0. Optical path, 1 cm.

— Oxidized
 ---- Reduced with ascorbic acid

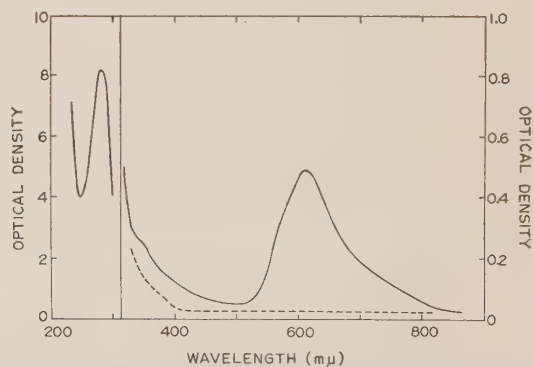


FIG. 7. Absorption spectra of *R. succedanea* laccase. 1.00% solution in 0.13 M phosphate buffer of pH 6.5. Optical path, 1 cm.

— Oxidized
 ---- Reduced with ascorbic acid

Copper and Other Constituents—Metal constituents in the ashes of both laccases were

analyzed by emission spectrography. Strong emission lines due to copper were detected, but no lines corresponding to other heavy metals could be observed. Besides copper, calcium and traces of magnesium were always detected in both of the enzyme preparations. X-ray fluorescence spectroscopic analysis of the ashes also indicated the presence of copper as the only heavy metal constituent.

The copper contents of the purified laccase preparations as measured by the dithizone method slightly varied from preparation to preparation in spite of the fact that the preparations, at least those of *R. vernicifera* laccase, employed were electrophoretically and ultracentrifugally pure (Table III).

In accordance with the rather low nitrogen contents of the laccase preparations (Table III), it was found that both enzymes contain considerable quantities of sugar constituents. Both hexoses and hexosamines were detected. The detection of hexoses was carried out by the thymol-sulfuric acid method (17) and that of hexosamines by Elson-Morgan reaction (18) after hydrolyzing the enzyme preparations by heating at 100°C for 5 hours with 4 *N* hydrochloric acid.

Molecular Weight—The partial specific volume of the two laccases was determined by measuring the specific gravity of their solution in 0.1 *M* phosphate buffer of pH 6.5. Ostwald-type picnometers were used, and the temperature was kept at 25°C. The concentration of enzymes was about 1.0 g. per 100 ml.

Sedimentation measurements were carried out with a Spinco model E analytical ultracentrifuge. Six sedimentation experiments for *R. vernicifera* laccase and two experiments for *R. succedanea* laccase were carried out with protein concentrations ranging from 0.3 to 1.2 per cent. The solvent used was 0.1 *M* phosphate buffer of pH 6.5. The measured values of sedimentation coefficient were corrected to 20°C in a solvent with the viscosity and density of water by the usual procedure. Since the sedimentation coefficient was considerably dependent on the protein concentration,

the value at infinite dilution was obtained by extrapolating to zero concentration.

Diffusion measurements were carried out in a Spinco model H electrophoresis apparatus at 4°C using 0.1 *M* phosphate buffer of pH 6.5 as solvent. Three diffusion measurements for *R. vernicifera* laccase and one measurement for *R. succedanea* laccase were carried out with protein concentrations ranging from 0.3 to 0.5 per cent. The diffusion coefficient was computed by the maximum ordinate—area method, and the values obtained were corrected to 20°C in a solvent with the viscosity of water by the usual procedure. No appreciable dependency on the protein concentration was observed with diffusion coefficient.

TABLE III
Copper Contents and Nitrogen Contents
of the Laccases

	<i>R. vernicifera</i> laccase	<i>R. succedanea</i> laccase
Copper contents	0.23–0.255% ¹⁾	0.22–0.240%
Nitrogen contents (micro-Kjeldahl method)	10.1%	8.8%

1) Based on the weight of enzymes dried at 110°C.

TABLE IV
Partial Specific Volume (*V*), Diffusion Coefficient (*D*_{20W}),
Sedimentation Coefficient (*S*_{20W}), and Molecular
Weight (*M*_{SD}) of the Two Laccases

	<i>R. vernicifera</i> laccase	<i>R. succedanea</i> laccase
<i>V</i> (ml. g. ⁻¹)	0.70	0.70
<i>D</i> _{20W} (cm. ² sec. ⁻¹)	3.57 × 10 ⁻⁷	3.9 × 10 ⁻⁷
<i>S</i> _{20W} (cm. sec. ⁻¹)	6.25 × 10 ⁻¹³	6.5 × 10 ⁻¹³
<i>M</i> _{SD}	1.41 × 10 ⁵	1.3 × 10 ⁵

Corrected values of the sedimentation coefficient and diffusion coefficient, and the partial specific volume of two laccases thus obtained were recorded in Table IV. The molecular weights of the two enzymes were

computed using these values obtained (Table IV). From the maximum values of copper-contents (Table III), the minimal molecular weight for the two enzymes can be calculated; 2.5×10^4 for *R. vernicifera* laccase and 2.6×10^4 for *R. succedanea* laccase are obtained. Comparing these values with M_{SD} -values, it may be concluded that the both laccases have 5 or 6 atoms of copper per molecule.

DISCUSSION

Considerable discrepancies can be cited among the properties of lacquer-tree laccases so far reported by various investigators. Tissi res (14) has purified a laccase from latex which he believed to have been obtained from *R. succedanea* and reported that the purified enzyme contained 0.25% copper, was most active at pH 7.1 in the oxidation of *p*-phenylenediamine, and had a Q_{O_2} value of 3×10^4 for the same substrate. Starting from latex of the same species of lacquer tree, however, D. Bertrand (7, 11) obtained a purified laccase preparation which, in contrast to Tissi res' enzyme, contained only 0.03% copper and had a pH optimum at pH 6.3 in the oxidation of *p*-phenylenediamine, though it possessed a relatively high Q_{O_2} value of 1×10^4 . These and other discrepancies among the reported properties have caused much confusion concerning the nature of lacquer-tree laccases.

Such discrepancies may, at least partly, become understandable if one assumes that different species of lacquer trees contain laccase of different properties. A second reason for the discrepancies may be sought in the difficulty in obtaining authentic specimens of lacquer latexes. As the production of lacquer latexes is restricted in south-eastern Asiatic countries, it is usually extremely difficult in the imported countries to obtain exact information on the species of lacquer tree from which the imported latex has been produced. Furthermore, it is often possible that latexes from different species of lacquer trees are mixed by exporters especially in those district where *R. vernicifera* and *R. succedanea* are cultivated together.

The present study is an attempt to further clarify these situations and was made possible by courtesy of Saito Co. Ltd. who kindly supplied us with carefully characterized specimens of authentic latexes obtained from different species of lacquer trees. Comparison was further made in the present study of the properties of highly purified laccase preparations obtained from Japanese and Indo-Chinese lacquer trees (*R. vernicifera* and *R. succedanea*, respectively). It was thus clearly established that the two laccases are definitely different in many respects. Namely, the enzyme from *R. succedanea* is much more catalytically active than that from *R. vernicifera*; the two enzymes have different pH optima and Michaelis constants which vary somewhat with the substrates employed; and they behave differently in ion-exchange chromatography. These findings appear to explain some of the discrepancies among the reported properties of lacquer-tree laccases.

In fact, it was noted that the pH optimum (7.4–7.6) of *R. vernicifera* laccase purified in this laboratory is in close agreement with those of the laccase preparations which Keilin and Mann (4, 6) and Tissi res (13, 14) claimed to have purified from latex of *R. succedanea*. It is very probable that these workers were supplied with *R. vernicifera* latex under the name of *R. succedanea* latex. The optimum pH of purified *R. succedanea* laccase in hydroquinone oxidation was found here to be pH 6.4 which is the same with that of D. Bertrand's laccase (7–12) prepared from the same species of lacquer tree. The very low copper content and relatively low Q_{O_2} of his preparation may be probably due to its insufficient purification.

Chinese lacquer tree is systematically classified as the same species with Japanese lacquer tree (*R. vernicifera*). It seemed, therefore, interesting to compare the properties of Chinese lacquer tree enzyme with those of Japanese lacquer tree enzyme. We have succeeded in purifying laccase from latex of Chinese lacquer tree produced in En Shih prefecture, China, by essentially the same pro-

cedures as used for Japanese lacquer tree enzyme. The purified enzyme was found to be identical with that from Japanese lacquer tree in all respects. No differences could be detected between the two preparations in catalytic activities (pH optima and Michaelis constants for hydroquinone and catechol, Q_{O_2} for hydroquinone), heat stability, and physico-chemical properties (absorption spectra and sedimentation coefficient).

It was also attempted to purify *M. usitata* laccase from latex of Burmese lacquer tree. The laccase content of this latex was, however, very low (Q_{O_2} of crude latex was about 3 which is much lower than those of *R. vernicifera* and *R. succedanea* latexes recorded in Table I), and it was not possible to obtain laccase preparations pure enough for the determinations of their physico-chemical properties. Nevertheless, the results obtained with crude enzyme preparations indicated that *M. usitata* laccase is also different in many properties from *R. vernicifera* and *R. succedanea* laccases. The pH optimum of *M. usitata* enzyme in hydroquinone oxidation was found to be as low as pH 4.5.

Although it is now clear that laccases of different species of lacquer trees represent different chemical entities, it is also important not to overlook similarities between *R. vernicifera* and *R. succedanea* enzyme. Both laccases contain nearly equal amounts of copper, show similar absorption spectra, and possess similar molecular weights. The catalytic mechanisms of the two laccases, therefore, appear to be similar to each other.

SUMMARY

1. Highly purified laccases were obtained by ion-exchange column chromatography from Japanese lacquer tree (*Rhus vernicifera*) and Indo-Chinese lacquer tree (*Rhus succedanea*). From comparative studies of the two enzymes, it was concluded that they represent different chemical entities.

2. The two laccases have different pH optima and Michaelis constants in the oxidation of hydroquinone, catechol, and L-ascorbic acid. *R. succedanea* laccase has a much higher

specific activity and heat stability than *R. vernicifera* laccase.

3. Both enzymes are strongly blue in color and contain nearly equal amounts of copper. Calcium and traces of magnesium can also be detected in the two enzymes. Their absorption spectra are closely similar to each other, though the positions of absorption maxima are slightly different in the two.

4. Molecular weights of both laccases calculated from their sedimentation coefficients, and partial specific volumes are nearly the same. The two enzymes both contain 5 or 6 atoms of copper per molecule.

The author wishes to express his hearty gratitude to the late Prof. A. Nishimura for his interest and encouragement during this investigation, and to Prof. Y. Ogura, University of Tokyo, for his valuable advices. Thanks are also due to Mr. S. Yamamoto, the Institute for Infectious Diseases, University of Tokyo, for sedimentation measurements, to Miss K. Watatsu, the Institute of Applied Microbiology, University of Tokyo, for the operation of Spinco model H electrophoresis apparatus, and to Misses F. Shimazaki and Y. Asahi for their co-operation in this work. The author also expresses his gratitude to Saito Co. Ltd. for the kind supply of lacquer latexes.

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Isolation and Identification of the Activator of Liver Tryptophan Pyrrolase

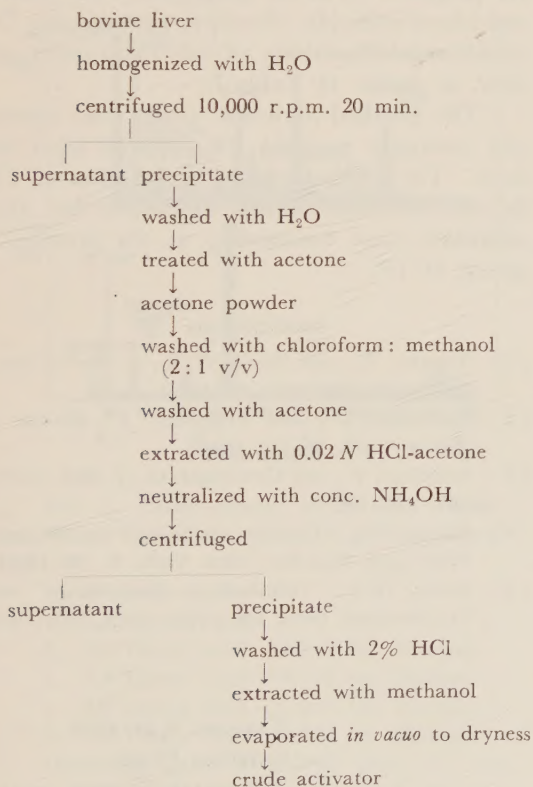
Tryptophan pyrrolase (TP), the enzyme catalyzing the oxidation of L-tryptophan to formylkynurenine, was partially purified and identified as an iron porphyrin protein (1). TP activity of rat liver homogenate was confined to the supernatant fraction obtained by centrifugation at $105,000\times g$ and the addition of isolated microsomal fraction was found to stimulate TP activity of supernatant. The activator of TP of microsomal fraction

TP activity as well as the activator of microsomal fraction (2, 3).

In this report, the activator of TP was isolated from bovine liver by means of silicic acid column chromatography and paper chromatography, and identified as protohemin IX. The activating effect of the sample to be tested on TP activity was estimated as follows. The liver of Wister King rats were homogenized in 9 vol. of 0.14 M KCl containing 0.0025 N NaOH and

SCHEME I

Isolation of the Activator of TP from Bovine Liver



could be extracted with 0.01 N HCl acetone and was a heat stable organic substance. Further, it was shown that hematin stimulated

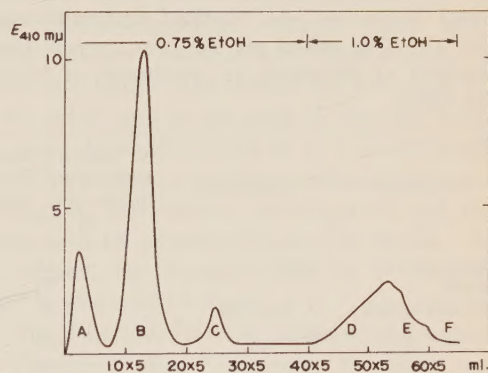


Fig. 1. Silicic acid column chromatography of the activator of TP.

The column was 2.5×60 cm, and contained 50 g. silicic acid. The eluant consisted of 0.75% ethanol in chloroform. The alcohol content of the eluant was increased to 1.0 per cent after 200 ml. had passed through. Five ml. fractions were collected and the absorbancy at the wave length of $410\text{ m}\mu$ was measured.

centrifuged at $105,000\times g$ for 60 minutes at 0°C . The supernatant was incubated with 0.003 M L-tryptophan, 0.04 M sodium phosphate buffer with the sample to be tested. After incubation for 60 minutes at 37°C ,

formed kynurenine was estimated spectrophotometrically (5). The isolation method of the crude activator was presented in Scheme I.

The crude activator was dissolved in chloroform and chromatographed on a silicic acid column. The typical pattern of the chromatogram was shown in Fig. 1.

The distribution of the activator of TP was examined in each fraction and as shown in Table I, the stimulatory activity was found in fraction D and E.

TABLE I

The Distribution of the Activator of TP

The liver of Wister-King rats were homogenized in 9 vol. 0.14 *M* KCl containing 0.0025 *N* NaOH and centrifuged at 105,000 $\times g$ for 60 min. at 0°C. The supernatant was incubated with 0.003 *M* L-tryptophan, 0.04 *M* sodium phosphate buffer with additions as indicated. After incubation for 60 min. at 37°C, formed kynurenine was estimated spectrophotometrically (5). Two μ moles per liter (final concentration), calculated as equivalent as protohemin, of fractions were added.

Treatment	Additions	TP activity (μ moles kynurenine formed hr./g., wet weight of liver)
Silicic acid column chromatography	none	1.13
	A fraction	1.36
	B "	1.30
	C "	1.27
	D "	2.84
	E "	2.43
Silicic acid chromatography and paper chromatography	R_f 0.45 fraction	2.00
	R_f 0.75 "	1.25
Protohemin IX (2 μ moles/liter)		2.02

For further purification, the paper chro-

matographic method using 8% glacial acetic acid in water-saturated toluene as solvent was employed and both the fractions of D and E showed two benzidine-hydrogen peroxide reactive spots of R_f 0.75 and R_f 0.45. The stimulatory activity was found only in the spot of R_f 0.45, as shown in Table I. 0.0014 mg. of the most purified activator had the same effect on TP activity of the supernatant of liver as about 150 mg. of microsome (dry weight), indicating that an approximately 10×10^4 fold purification was achieved. This substance was indentified as protohemin IX from paper chromatography using toluene-acetic acid system and pyridine-isopropyl alcohol-8 *N* ammonium hydroxide system (1:2:1) and by the properties of visible spectra of TP activator in comparison with the crystallized protohemin IX prepared from the hemoglobin of human red blood cells (4). Further, protohemin IX stimulated TP activity of the supernatant of liver, as shown in Table I.

The purified activator could also stimulate partially purified TP isolated from rat liver. The mechanism of stimulation of TP by the activator is still unknown, but this substance may be serving as the prosthetic group of TP.

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Occurrence of 3', 5'-cyclic AMP in Milk

Denamur *et al.* (1) previously reported that sheep milk has a unique nucleotides composition quite different from those of ordinary mammalian tissues, demonstrating the predominant contents of UDP-sugars. We have had much interest in the problem of infant nutrition and undertaken several comparative studies on the nucleotides of human and cow's milk and colostrum. The procedures for the analysis of acid soluble nucleotides are essentially the same as those of Hurlbert *et al.* (2) and of Terada (3). Each peak thus ob-

tained was further subjected to the rechromatography with Dowex-1, (Am-F system), previous to the subsequent paper chromatographic and electrophoretic identifications. Two remarkable features were obtained from the experiments. One is a quite distinct difference in the nucleotide patterns between human and cow's milk (Fig. 1). The other is the fact that human milk and colostrum have similar nucleotide constituents, contrary to those of cow's milk, which is simpler in its composition and less in its content than those of the colostrum. Cow's colostrum resembles to sheep's milk in its nucleotide content. In these chromatographic studies there were found several unidentified constituents, of which the major one found in the peak III (cow's milk) and in the peak IV (human milk) has now been identified as 3', 5'-cyclic AMP (4). The nature of another unidentified components is now under investigation and the results will be presented later in detail. In this report, the identification of 3', 5'-cyclic AMP is described below.

The fraction III was rechromatographed on Dowex-1, (Am-F system), to yield one major component III-2 with a small amount of a concomitant III-1. The base moiety of the component III-2 was proved to be adenine by ultraviolet spectroscopy and by paper chromatographies with five different solvent systems after hydrolysis (12 *N* perchloric acid, 100°C, 1 hour). The molar ratio of the base (U.V.), ribose (Orcinol test), total and labile phosphorus (Fiske-SubbaRow) was found to be 1:0.89:0.94:0.00. The component was demonstrated to be quite stable to 0.02 *N* perchloric acid and 0.1 *N* KOH treatments (100°C, 180 minutes), and also extremely resistant to the enzymatic digestions with 5'-nucleotidase (bull semen), phosphomonoesterase (human prostate) and snake venom phosphodiesterase, although it liberated a

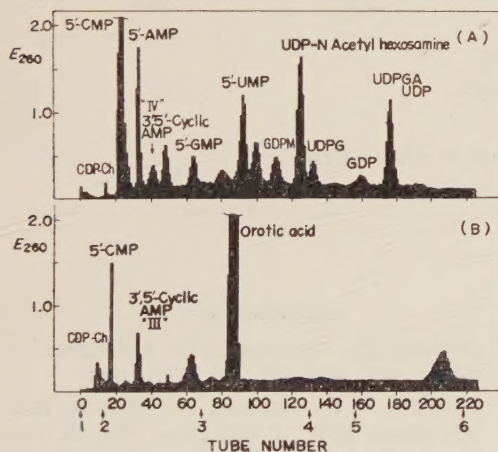


FIG. 1. Chromatogram of the acid soluble nucleotides from human milk (A) and from cow's milk (B).

1. *N* Formic Acid
2. 4 *N* Formic Acid
3. 4 *N* Formic Acid + 0.2 *M* Am. Formate
4. 4 *N* Formic Acid + 0.4 *M* Am. Formate
5. 4 *N* Formic Acid + 0.8 *M* Am. Formate
6. 4 *N* Formic Acid + 2.0 *M* Am. Formate

Overscaled parts of 5'-CMP peak (A) are 33.00, 26.58, and of orotic acid peak (B) are 5.01, 11.31, 19.14, 22.95, 11.37 respectively.

Abbreviations: *i*-PrOH, *iso*-propanol; NH₄CAC, ammonium acetate; NaOAc, sodium acetate.

minute amount of 3'- or 5'-AMP very slowly with the last enzyme. The component migrated as fast as AMP in paper electrophoresis at PH 4.15, but only half of AMP at PH 7.5. The component was not oxidized by periodate.

Finally the component was conclusively identified by comparison with an authentic

ished, we met the report of Deutsch *et al.* (7) on the nucleotides composition of human milk. Although their results generally agree with ours, several discrepancies existed. Namely, we failed to find, 3'-AMP or 2'- (3'-) UMP, and they did not mention about the existence of 3', 5'-cyclic AMP.

TABLE I
Paperchromatography and Paperelectrophoresis of "III-2"

R_f in paperchromatography	5'-AMP	3', 5'-cyclic AMP	"III-2"
Solvent 4 (asc.)	0.030	0.075	0.072
Solvent 6 (desc.)	0.47	0.76	0.76
Solvent 7 (desc.)	0.75	0.76	0.76
Solvent 8 (asc.)	0.49	0.56	0.56
Solvent 9 (asc.)	0.41	0.15	0.15
Electrophoresis mobility (cm.) 0.05 M Borate Buffer pH 9.2 70 volt./cm. 50 min.	19.0	13.3	13.6
Solv. 4 <i>n</i> -BuOH, water, HCOOH, (77:13:10) ^{a)}			
Solv. 6 95% EtOH, NH ₄ OAc (pH 7.5), (75:30) ^{a)}			
Solv. 7 <i>i</i> -PrOH, water, (7:3) ^{a)}			
Solv. 8 <i>i</i> -PrOH 170 ml., conc. HCl 41 ml., water to make 250 ml.			
Solv. 9 sat(NH ₄) ₂ SO ₄ , <i>i</i> -PrOH, M NaOAc (80:2:20) ^{a)}			

a) v/v. Toyo Filter Paper No. 51 A was used.

sample of 3', 5'-cyclic AMP (synthesized chemically by the method of Smith *et al.* (5)) by paper chromatography, electrophoresis (Table I) and infrared spectroscopy. The contents of this compound in cow's colostrum, cow's milk, human colostrum and human milk, estimated on the basis of ultraviolet absorption ($E_{260}=1.43 \times 10^4$), were about 0.80, 0.35, 0.08 and 0.08 μ moles/100 ml. respectively.

The occurrence of 3', 5'-cyclic AMP in milk, especially in cow's milk lacking in 5'-AMP, might be of some biological significance, taking consideration of its known biological actions (6).

When this manuscript had been just fin-

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